STUDIES ON THE ISOLATION OF MURINE AND OVINE EMBRYONIC STEM CELLS

DAVID NORMAN WELLS

BAgSc(Hons) (Lincoln University, New Zealand)

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Department of Reproduction and Development,
Agricultural and Food Research Council,
Institute of Animal Physiology and Genetics Research,
Roslin,
Midlothian EH25 9PS,
Scotland
and
Department of Genetics
University of Edinburgh

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To Bubbles

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ABSTRACT

Techniques for the isolation and genetic manipulation of embryonic stem cells (ES cells) in the mouse are well established. However, little is understood regarding the mechanisms which enable pluripotent cells from the early mouse embryo to be diverted experimentally from their normal fate of differentiation *in vivo* and maintained instead in culture, as stable ES cell lines. Such knowledge might aid the establishment of ES cells from other mammalian species; particularly from farm animals, with the potential applications of genetically manipulating the stem cells to modify production traits. The objective of this thesis was to investigate various factors which may influence the potential of murine and ovine embryos to yield ES cells.

Mouse genotype influences the capacity of embryos to give rise to ES cells. A significantly greater proportion of day 3.5 *p.c.* mouse embryos from the inbred 129/Sv-*CP* strain yielded ES cell lines compared to crossbred F_2 (C57BL/6 X CBA/Ca) embryos. The effect of mouse genotype was first observed in the significantly greater proportion of 129/Sv-*CP* embryos that gave rise to ES-like colonies at the first passage, following the disaggregation of inner cell mass (ICM) outgrowths. The efficiency of ES cell isolation was significantly increased by culturing murine blastocyst-stage embryos which had been induced experimentally, to enter a period of implantational delay for five days.

Typically, murine ES cells have been derived from the day 3.5 *p.c.* ICM, although day 2.5 *p.c.* morulae have also been used. Here, a study describes the isolation of (at least) tripotent ES cell lines from the primitive ectoderm of day 5.5 *p.c.* egg cylinder-stage mouse embryos. Although this study has shown that in the mouse, primitive ectodermal cells influenced by endoderm can be established as ES cells in culture, the frequency is significantly lower than from the ICM. ES cells isolated from the three different embryonic stages, equivalent to three days of development, have the same morphological characteristics.

The brief exposure of day 3.5 *p.c.* embryos from the 129/Sv-*CP* mouse strain to treatments expected to perturb gene expression, heat shock or puromycin-containing medium, significantly increased the frequency of ES cell isolation. These effects resulted from increases in the proportion of embryos giving rise to ES-like colonies at the first passage which, in the case of heat shocked embryos, were also less likely to differentiate in subsequent passages. ES cell lines derived from embryos exposed to heat shock or puromycin have retained the capacity for *in vivo* development, as the stem cells colonised the germline in some chimaeric mice. These results suggest that

the isolation of ES cells depends upon reversible, epigenetic changes in the pattern of gene expression and that the heat shock and puromycin treatments utilised here, may have helped to promote these changes *in vitro*. It is suggested that heat shock and implantational delay may act through a similar mechanism; whereby the treatments may have interfered with the transcriptional activation of genes responsible for differentiation of the ICM.

Carbohydrate antigens which are expressed in a stage- and/or tissue-specific manner, have been detected on the cell surface of pre-implantation sheep embryos. Of the 11 antibodies studied, however, none detected antigens specific for the ICM or primitive ectoderm, which could have been utilised as possible lineage markers for pluripotent cells, *e.g.* ovine ES cells. Antibodies specific for trophectodermal and endodermal antigens may be useful in selectively lysing differentiated cells in sheep stem cell cultures.

Studies directed towards the isolation of permanent stem cell lines from sheep embryos were not successful in maintaining cells possessing an ES-like morphology for more than three passages in culture, before the cells either died or differentiated. In a comparison of six to nine day old embryos, day nine micro-dissected embryonic discs gave rise to the highest proportion of ES-like outgrowths. In addition, these day nine outgrowths yielded more first passage colonies, which maintained a stable morphology (slightly) longer. Medium containing 4% Ultroser and 5% FCS or, supplemented with PDGF and insulin, may have some limited effects on stimulating proliferation and/or maintaining a more stable ES-like cell morphology. Sheep embryos treated with heat shock or puromycin did not yield stable cultures of ES-like cells. The difficulties experienced in the isolation of stem cells from the sheep compared to the mouse, are due possibly to fundamental species differences in specific culture requirements and/or embryology.

The studies described in this thesis have shown a better understanding of some of the factors which influence the capacity of murine embryos to yield permanent ES cell lines. These factors may also have implications for the isolation of ES cells from the sheep (and other species).

PUBLICATIONS AND AWARDS ARISING FROM THIS THESIS

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ABBREVIATIONS

BRL	Buffalo rat liver cells
DIA	stem cell differentiation inhibiting activity
DMEM	Dulbecco's Modification of Eagle's Medium
$DMEM_{10}$	DMEM + 5% NCS + 5% FCS
EC	embryonal carcinoma cells
EDTA	ethylenediamine-tetraacetic acid
EGTA	ethyleneglycol-tetraacetic acid
ES	embryonic stem cells
ES_{US}	ES cell medium containing 4% Ultroser
ES_{US+FCS}	ES cell medium containing 4% Ultroser + 5% FCS
ES_{10}	ES cell medium containing 5% NCS + 5% FCS
ES_{15}	ES cell medium containing 5% NCS + 10% FCS
ES_{20}	ES cell medium containing 10% NCS + 10% FCS
FCS	foetal calf serum
GPI	glucose phosphate isomerase
HSE	heat shock element
HSP	heat shock protein
HSTF	heat shock transcription factor
ICM	inner cell mass
LIF	myeloid leukemia inhibitory factor
LND I	lacto-*N*-difucohexaose I
LNF I	lacto-*N*-fucopentaose I
LNF II	lacto-*N*-fucopentaose II
LNF III	lacto-*N*-fucopentaose III
LNnD I	lacto-*N*-neo-difucohexaose I
LNnF I	lacto-*N*-neo-fucopentaose I
LNT	lacto-*N*-tetraose
mAb	monoclonal antibody
NCS	newborn calf serum
PB1	enriched phosphate buffered saline (with Ca^{2+} and Mg^{2+})
PBS	phosphate buffered saline (w/o Ca^{2+} and Mg^{2+}, unless otherwise stated)
p.c.	*post coitum* (in this thesis, the morning of detection of the copulation plug in the mouse is set at day 0.5 *p.c.*)
p.o.	*post oestrus*
STO	a permanent mouse fibroblast, "feeder cell" line
TED	trypsin and EDTA solution
TEG	trypsin and EGTA solution

Common Suppliers of Experimental Consumables

BDH Ltd. - Thornliebank, Glasgow G46 7TP

Calbiochem (c/o Novabiochem Ltd.) - Nottingham NG7 2QJ

CAMLAB Ltd. - Cambridge CB4 1TH

Clark Electromedical Instruments Ltd. - Pangbourne, Reading RG8 7HU

Difco Laboratories Ltd. - East Molesey, Surrey KT8 0SE

Ethicon Ltd. - Edinburgh EH11 4HE

Flow Laboratories Ltd. - Rickmansworth, Hertfordshire WD3 1PQ

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CHAPTER 1

LITERATURE REVIEW

Within the early mammalian embryo there exists for a brief period, a population of undifferentiated, pluripotent cells from which all the tissues of the foetus, including the germline, will develop. Two procedures have been utilised to divert pluripotent cells, from early mouse embryos, from their normal fate of differentiation and to establish them instead into tissue-culture as permanent, undifferentiated cell lines. Historically, the first pluripotent cells to be isolated were the embryonal carcinoma (EC) cell lines derived from teratocarcinomas and then later, the embryo-derived embryonic stem (ES) cell lines. These two cell types have many characteristics in common to those of pluripotent cells within the embryo itself, the major difference being that, under suitable culture conditions, these cell lines have the capacity for continued proliferation. However, when re-introduced into an early embryo, these stem cells retain the capacity to participate in normal *in vivo* development.

This literature review describes the presence of these pluripotent cells within the early mouse embryo, their isolation into culture as EC or ES cells and the relationships between the three cell types. A section briefly reviews some of the applications of stem cells in the study of early development, including the exciting experiments involved with introducing precise genetic modifications into ES cells *in vitro*; with the aim of creating new mouse mutants, enabling the study of gene expression and function during *in vivo* development. A final section is devoted to the recent attempts to isolate these stem cells in species other than the mouse.

1.1 PLURIPOTENT CELLS IN THE EARLY MURINE EMBRYO

The orderly pattern of cell division and differentiation during early mammalian embryo development results in embryonic cells becoming progressively more specialised and allocated to form the "body plan" of the foetus. Studies utilising chimaeras, produced from genetically dissimilar pre-implantation embryos, have identified cells within the early murine embryo which are progenitors of subsequent tissues of the conceptus and form cell lineages (Gardner, 1985; Gardner and Beddington, 1988). The developmental potential of these progenitor cells may be classified as either totipotent (capable of contributing to all embryonic and extra-embryonic, placental tissues of the conceptus), pluripotent (capable of contributing to all foetal tissues), or multipotent (contributing to only a few cell types).

Because of the constant change in the developmental potential of embryonic cells, the mammalian embryo possesses a totipotent/pluripotent cell population for a period of only a few days, before these cells all progressively differentiate to form the tissues of the developing foetus. This cell population is often referred to as "stem" cells; however, in embryology, this does not mean that these cells are capable of indefinite self-renewal *in vivo*, but simply that they are the "progenitor" cells of the subsequent foetal tissues (Gardner and Beddington, 1988).

Following fertilisation in the mouse, all of the blastomeres up to the 16-cell-stage are potentially totipotent (Kelly, 1977; Ziomek *et al.*, 1982a). Single blastomeres from two-cell-stage embryos have given rise to adult mice (Tarkowski, 1959). However, individual blastomeres from four-cell mouse embryos are probably unable to form viable offspring, because of the low number of cells in the inner cell mass (ICM) of resultant blastocysts (Kelly, 1977). When combined with genetically marked cells, single blastomeres from four-, eight- (Kelly, 1977) and 16-cell embryos (Ziomek *et al.*, 1982a) are able to contribute to all tissues of the conceptus; that is, they are totipotent.

Although the potential of blastomeres appears not to be restricted during cleavage, events in the non-manipulated eight- to 16-cell embryo result in the first differentiation of cells into two divergent cell lineages: the trophectoderm and the ICM. This differentiative process commences with compaction, where the blastomeres increase cellular contact and become a tight mass of cells. This is then followed by cell polarisation, generating a population of six to eight inner (apolar) cells, which will form the ICM, and eight to 10 outer (polar) cells, which will form the trophectoderm of the so-called blastocyst-stage embryo (Ziomek *et al.*, 1982b). Cell lability may persist beyond the time of normal lineage allocation in the embryo, as early ICMs have the capacity to form trophectoderm derivatives in chimaeric conceptuses (Rossant and Lis, 1979).

The first differentiative event ends on day 3.5 *post coitum* (day 0.5 *p.c.* is the morning of detection of the copulation plug) with the formation of a fully expanded, fluid-filled blastocyst consisting of an outer spherical layer of trophectoderm (which is multipotent, forming some tissues of the placenta) and a small group of around 15-20 ICM cells at one pole of the embryo. At this stage, the cells of the ICM are only pluripotent as they have lost the potential to form trophectoderm (Gardner, 1985).

The next differentiative event is completed by 4.5 days *p.c.* (just prior to implantation) and involves the delamination of a primitive endoderm (or hypoblast) layer from the blastocoelic surface of the ICM. The remaining ICM is termed the primitive ectoderm (or epiblast) and colonises the foetus, extra-embryonic mesoderm and amniotic ectoderm - but not the extra-embryonic endodermal lineages (Gardner

and Rossant, 1979; Gardner, 1982). The findings of Gardner and co-workers (1985) have suggested that the majority of cells from the day 4.5 *p.c.* primitive ectoderm may all be equally pluripotent.

As the cells of the trophectodermal and primitive endodermal lineages establish the implantation site , the primitive ectoderm proliferates rapidly, forming a pseudo-stratified epithelium, with no morphological signs of differentiation in the day 6.5 *p.c.* egg cylinder-stage embryo (Gardner and Beddington, 1988). Indirect methods have been utilised to study the fate of cells from post-implantation embryos, as there are technical difficulties in performing *in vivo* chimaeric analyses on such advanced developmental stages. The production of offspring following the destruction of 85% of the day 6.5 to 7.0 *p.c.* primitive ectoderm, has suggested that the allocation of cells to different lineages within the epiblast is unlikely (Snow and Tam, 1979). The short-term *in vitro* experiments conducted by Beddington (1982), where regions of the day 7.5 *p.c.* primitive ectoderm were grafted into heterotopic positions in synchronous host embryos, revealed that the posterior and distal regions of the primitive ectoderm were developmentally labile and not restricted to their normal fate. This was compatible with the concept that cell position plays an important role in the fate of cells colonising a particular lineage (Tarkowski and Wroblewska, 1967). The anterior region of the primitive ectoderm, in contrast, was shown by day 7.5 *p.c.* to have a strong preference to form the definitive ectoderm of the foetus (Beddington, 1982). This marks the beginning of the overt differentiation of the primitive ectoderm into the extra-embryonic mesoderm and the definitive layers of the foetus itself - the embryonic ectoderm, endoderm and mesoderm. Hence, the loss of pluripotent cells from the mouse embryo occurs after day 7.5 *p.c.*.

The germ cells are another source of potentially totipotent cells. In the mouse, the germ cell progenitors are not apparent until day 7.5 *p.c.* and are derived from the primitive ectoderm (Gardner *et al.*, 1985; Ginsburg, Snow and McLaren, 1990). The germ cells themselves have restricted potential forming very specialised gametes. However, following fertilisation totipotency is restored to the zygote.

1.2 MURINE EMBRYONAL CARCINOMA CELL LINES

Spontaneous teratocarcinomas are tumours that most commonly occur in humans and certain inbred strains of mice and may occur in the gonads of both males and females. Teratocarcinomas may be composed of a chaotic array of many kinds of cells and tissues in various stages of maturation (Stevens and Hummel, 1957). Their stem cells, embryonal carcinoma (EC) cells, maintain the malignancy of the tumour, self-renewing the stem cell population and are pluripotent, differentiating to form the

cellular derivatives of the three primary germ layers - embryonic ectoderm, endoderm and mesoderm (Kleinsmith and Pierce, 1964). When all of the EC cells differentiate, the tumours become benign and are instead called teratomas. Unless specifically qualified, both types of tumour have been included in the general term "teratoma" in this thesis.

This section reviews some of the factors which may influence the development of spontaneous and experimentally induced murine teratocarcinomas (from either male primordial germ cells or from early embryos) and the *in vitro* isolation and developmental characteristics of EC cells, derived from these tumours.

1.2.1 Testicular Teratomas

In mice, congenital testicular teratomas were first observed in approximately 1% of the males from a line of mice of the inbred 129 strain, subsequently designated 129/Sv (Stevens and Little, 1954). A number of genetic loci have been found to influence the susceptibility of mice to testicular teratomas. The introduction of the mutant Steel gene (Sl^J) onto the 129/Sv genetic background, markedly increased the incidence of spontaneous teratomas (to around 7%: Stevens and Mackensen, 1961). The males of a subline of mice (129/*ter*Sv) congeneic with 129/Sv have a high (30%) spontaneous incidence of congenital testicular teratomas, thought to have been due to a single gene mutation (Stevens, 1973). The presence of the A^y allele at the agouti locus has been shown to decrease the incidence of testicular teratomas (Stevens, 1975).

Spontaneous testicular teratomas originate from the foetal primordial germ cells within the seminiferous tubules (Stevens, 1962). Abnormal proliferation of the primordial germ cells leads to the formation of embryonic ectoderm-like structures, which subsequently become disorganised and form tumours (Stevens, 1983). Indeed, Pierce and Beals (1964) have noted the similarities in the ultrastructural morphology between primordial germ cells and EC cells.

Testicular teratomas may be induced experimentally in some strains of mice (particularly strains 129/Sv-Sl^J and A/HeJ; Stevens, 1970a) by grafting (male) germinal ridges from day 12.5 to 13.5 *p.c.* foetuses into the testes of histocompatible adults (Stevens, 1964). The sex of the germinal ridge from day 12.5 *p.c.* foetuses cannot be morphologically determined and so half of the grafts developed into ovaries which did not contain teratomas (Stevens, 1964), presumably because the female primordial germ cells had entered meiosis (Monk and McLaren, 1981).

The site of the graft appears to be important When germinal ridges were transplanted to abdominal sites, although they developed into testes, few of them

contained teratomas (Stevens, 1970b). Subsequently, Stevens (1975) demonstrated that the lower temperature of the scrotum enhanced the induction of teratocarcinogenesis. Age of the germinal ridge was also of importance. When day 14.5 *p.c.* germinal ridges were grafted, very few teratomas developed, possibly indicating that the diploid male primordial germ cells had matured to a point where they were less susceptible to tumour induction (Stevens, 1970a).

1.2.2 Ovarian Teratomas

Ovarian teratomas originate from post-first-meiotic division oocytes within the ovary that have undergone spontaneous parthenogenetic activation *in situ* (Eppig *et al.*, 1977). They may develop into apparently normal early egg cylinder-stage embryos before becoming disorganised (Stevens and Varnum, 1974). This disorganisation may disrupt the normal cellular relationships within the embryo and allow the undifferentiated cells to proliferate further, or terminally differentiate (Stevens, 1970c). Half of the females from the LT/Sv strain of mice may develop this spontaneous form of ovarian tumour (Stevens and Varnum, 1974).

1.2.3 Embryo-Derived Teratomas

Experimentally, benign teratomas have been readily produced in various inbred lines from some animal species, by transplanting embryos to extra-uterine sites. Embryo-derived teratomas have been studied in mice (Stevens, 1970c), rats (Skreb, Svajger and Levak-Svajger, 1971) and hamster (Damjanov, 1978). Theoretically, it should be possible to generate similar tumours in other animal species if inbred lines are available. However, malignant teratocarcinomas have only been produced efficiently in inbred mouse strains.

The factors influencing the proportion of embryos giving rise to teratocarcinomas may be divided into embryo- and host-related factors.

1.2.3.1 Embryo-Related Factors

Ectopic transplantation of mouse embryos between the two-cell-stage and day 7.5 *p.c.* of development may give rise to a teratocarcinoma, whereas older embryos give rise only to teratomas (Stevens, 1968, 1970c; Solter, Skreb and Damjanov, 1970; Damjanov, Solter and Skreb, 1971). This upper limit presumably

corresponds to the loss of pluripotent stem cell progenitors after day 7.5 *p.c.*. Pre-implantation embryos develop within the ectopic site into structures resembling the egg cylinder-stage embryo, before becoming disorganised (Stevens, 1968). Diwan and Stevens (1976) have shown that only the primitive ectoderm tissue of the embryo was responsible for generating the teratoma. On average, less than 5% of pre-implantation-stage embryos transferred ectopically yield teratocarcinomas (due to the poor embryonic development within the ectopic site), compared to around 50% achieved with post-implantation stages (Stevens, 1968; 1970c).

Whilst embryo-derived teratocarcinomas can be produced in most, if not all, inbred mouse strains, the ratio of benign to malignant tumours may vary from one inbred strain to another. Utilising day 7.5 *p.c.* embryos transplanted beneath the kidney capsule, the range may be from 3:7 in BALB/c mice, 1:1 in C3H mice and to 9:1 in C57BL/6 mice (Damjanov, Bagasra and Solter, 1983). Mouse strains in which more than 50% of the embryos grafted to an ectopic site yield malignant tumours, are termed "teratocarcinoma-permissive" strains and include A/J, BALB/cJ, DBA/2J, CBA/J and C3H/J mice (Solter, Dominis and Damjanov, 1979). Low yield strains such as C57BL/6J and AKR/J, are termed "teratocarcinoma-nonpermissive". The nature of this permissiveness is not clearly understood, but may have genetic and epigenetic components which reside in both the embryo and the graft-receiving adult recipient.

Utilising reciprocal F_1 embryos from various inbred mouse strains, Damjanov and Solter (1982) demonstrated that some crosses between teratocarcinoma-permissive and nonpermissive parents yielded significantly more malignant tumours than embryos from the nonpermissive strain; but only when the mother of the F_1 hybrid embryo was of a permissive strain. This indicated the presence of some unknown maternal effect operating on the embryo.

1.2.3.2 Host-Related Factors

Embryo-derived teratocarcinogenesis depends upon the genetic background of the adult recipient and in some mouse strains, on the sex of the recipient also. Data from transplantation studies have clearly shown that embryos from some nonpermissive strains are not genetically determined to form benign teratomas, as they may develop into teratocarcinomas at a high frequency if transplanted into an appropriate histocompatible F_1 host, as opposed to the syngeneic recipient (Solter, Dominis and Damjanov, 1981). However, this hybrid stimulation does not occur in all F_1 combinations and there appears to be an epigenetic maternal factor involved, with a tendency for a higher response to teratocarcinogenesis if the

mother of the F_1 host is from a permissive strain (Solter *et al.*, 1981). Interestingly, males of the 129/J strain were significantly more teratocarcinoma-permissive, compared to when syngeneic embryos were grafted to 129/J females (Solter *et al.*, 1979).

Other approaches to increase the proportion of teratocarcinomas experimentally, including the use of allogeneic recipients, since early embryos do not express histocompatibility antigens (H-2) (Damjanov, Damjanov and Solter, 1987) and utilising immunocompromised mice as recipients (Damjanov *et al.*, 1983), have not been successful in overcoming the nonpermissiveness of certain inbred strains of mice. Conversely, when mice immunised with EC cells or mice bearing re-transplantable tumours (see section 1.2.4) were used as hosts, the incidence of teratocarcinomas significantly increased (Damjanov *et al.*, 1987). Thus, it appears that a normal or specifically stimulated immune response may be beneficial for development of embryo-derived teratocarcinomas.

1.2.4 Re-Transplantable Teratocarcinomas

Many testicular, ovarian and embryo-derived teratomas are benign and as a consequence fail to proliferate further following subsequent transplantation into a new host. This is also the commonest fate of primary malignant teratomas upon transfer. Occasionally, teratocarcinomas may continue to grow in histocompatible hosts after subcutaneous, or intramuscular, transfer of minced primary tumours. Re-transplantable teratocarcinomas have been established from spontaneous testicular and ovarian tumours and from embryo-derived tumours induced experimentally (reviewed by Stevens, 1983). Re-transplantable tumours can be further passaged by periodic re-transplantation and maintained as *in vivo* cell lines for several years in some instances (Stevens, 1958). During early transplant generations, this re-transplanting procedure generally favours the growth of the more malignant EC cells at the expense of the differentiated somatic tissues. However, with extended tumour generations, the stem cells may eventually become restricted in their developmental potency and may terminally differentiate into one or more cell types (Stevens, 1958).

Some re-transplantable teratocarcinomas may also be passaged by intraperitoneal injection of finely minced fragments. Occasionally, this leads to the formation of multiple tumours which grow wherever the cells implant within the body cavity. Frequently, however, the injected cells continue to grow as ascitic tumours (Stevens, 1959). Ascites forms grow either as single-cells or may form aggregates resembling six day old mouse embryos, which float freely in the ascites fluid. These so-called "embryoid bodies" may be composed of a core of EC cells surrounded by a

layer of endoderm-like cells or they may become fluid-filled and cystic. The inner stem cells of these embryoid bodies may remain undetermined for years, yet when they are transplanted subcutaneously they may give rise to teratocarcinomas composed of many different cell types (Pierce and Dixon, 1959).

1.2.5 Establishment of Embryonal Carcinoma Cell Lines

EC cells can be propagated for a number of years within the tumour environment (either in solid or ascites form), however, it is generally a more difficult task to recover the stem cells into tissue-culture. Cells with the typical undifferentiated EC morphology (small rounded cells lacking any specialised structures and possessing a large nuclear to cytoplasmic ratio, with one or more prominent nucleoli; Martin, 1975) have been established in culture from both solid tumours (Evans, 1972) and ascitic embryoid bodies (Kahan and Ephrussi, 1970). Briefly, the technique demands the disaggregation of tumours into small cellular clumps which are explanted into complex bicarbonate-buffered tissue-culture medium, with subsequent clonal selection of cells possessing an undifferentiated morphology. The co-culture of the disaggregated tumour cells with mitotically inactivated feeder cell layers may facilitate the establishment and maintenance of EC cell lines (Martin and Evans, 1975). However, EC cells may remain feeder-dependent as a result (Martin, 1975).

Teratocarcinomas which have been passaged several times *in vivo* contain a higher proportion of actively growing malignant cells (see section 1.2.4). So, the isolation of EC cells has been more successful with such re-transplantable tumours. Few *in vitro* EC cell lines have been established from primary tumours (Mintz and Cronmiller, 1981; McBurney and Rogers, 1982).

The majority of EC cell lines that have been isolated into culture have their origins in embryo-derived teratocarcinomas. However, all EC cell lines can be traced back to only some 16 independently derived tumours (a comprehensive listing of EC cell lines is to be found in Appendix I of Silver, Martin and Strickland, 1983; and reviewed by Robertson and Bradley, 1986). A smaller number of *in vitro* EC cell lines have been isolated from spontaneously occurring teratocarcinomas.

The different EC cell lines, although superficially resembling one another in basic morphology and in expression of cell surface antigens, compose a very heterogeneous set of tissue-culture lines (Robertson and Bradley, 1986). There is particular variability in their *in vitro* growth characteristics and requirements, chromosome constitution and differentiation ability as reviewed in the following sub-sections.

1.2.6 Differentiation of Embryonal Carcinoma Cells *In Vitro*

There are great differences between EC cell lines in their capacity to differentiate *in vitro* (Martin, 1975). Cell lines that show the most extreme type of restriction, known as "nullipotency", appear incapable of spontaneous differentiation. Other EC cell lines may be multipotent or pluripotent, reflecting the range of cell lineages present in the differentiated cell population. Generally, feeder-dependent EC cell lines are capable of differentiating into a larger range of different cell types than feeder-independent cell lines (Heath, 1983).

Spontaneous differentiation into a variety of cell types can be induced by either allowing cultures to become over-confluent, removing feeder cells in feeder-dependent cell lines, seeding cells at low plating density following passage, or by lowering the serum concentration (Rudnicki and McBurney, 1987). All of these methods are of little application in studying mechanisms of differentiation. Methods including the suspension culture of cellular aggregates and the addition of various exogenous drug promoters, induce more predictable and synchronous differentiation.

Some EC cell lines develop into embryoid bodies in suspension culture, where they consist of an inner core of EC cells surrounded by a layer of endodermal cells which secrete a mucopolysaccharide layer known as Reichert's membrane (Martin and Evans, 1975). This is analogous to the early events of differentiation in the mouse embryo and provides a model system for studying differentiation during the early post-implantation period. When these embryoid bodies attach to a tissue-culture surface, they differentiate extensively into cell types representative of the three primary germ layers; indicating such EC cell lines to be pluripotent (Martin, 1975).

The study of the cellular mechanisms involved in differentiation has been aided by certain chemical inducers promoting EC cells to differentiate into a specific cell type. For example, the addition of low concentrations of retinoic acid to monolayer cultures of EC cells from the nullipotent F9 cell line, stimulated the formation of parietal extra-embryonic endoderm cells (Strickland and Mahdavi, 1978); while exposure of cellular aggregates of F9 EC cells to retinoic acid resulted in the induction of visceral endoderm instead (Hogan, Taylor and Adamson, 1981). There is heterogeneity between different EC cell lines in their response to the same drug. For instance, aggregates of EC cells from the P19 cell line differentiated into neuronal cells in response to retinoic acid (McBurney *et al.*, 1982). Other compounds, such as dimethyl sulphoxide, induce some EC cell lines to form cardiac and skeletal muscle (McBurney *et al.*, 1982).

1.2.7 Chromosomal Characteristics of Embryonal Carcinoma Cells

EC cell lines are also heterogeneous with respect to chromosome complement. The majority of cell lines are aneuploid (abnormal chromosome number or structure); frequently with the loss of one sex chromosome (X or Y) and/or possessing various trisomic abnormalities. These karyotypic abnormalities may be due to the time EC cells persist within the tumour and may be further accentuated by the long term maintenance of cell lines *in vitro*.

Cytogenetic studies have shown the majority of primary embryo-derived tumours to have a normal modal chromosome number of 40 (Martin, 1975). With the increasing passage generation of transplantable teratocarcinomas, two well reported effects are observed: the restriction of differentiative capacity (Stevens, 1958) and the aquisition of karyotypic alterations (Iles and Evans, 1977). This can occur within the first few passages *in vivo* and is generally more severe in ascitic rather than solid tumours (Iles and Evans, 1977).

Only five completely euploid EC cell lines have been established (as listed in Silver *et al.*, 1983; and reviewed by Robertson and Bradley, 1986), namely OTT 6050 maintained transiently *in vivo* (Cronmiller and Mintz, 1978), C145b (Papaioannou *et al.*, 1979), P10 (McBurney and Strutt, 1980), METT-1 (Mintz and Cronmiller, 1981) and P19 (McBurney and Rogers, 1982). It is important to note that of these cell culture lines, two, P19 and METT-1, were isolated from primary teratocarcinomas without serial transplantation. The prerequisite of a normal euploid karyotype cannot be over-emphasised if EC cells are to result in germline transmission of the tumour cell genotype in chimaeric animals.

1.2.8 Differentiation of Embryonal Carcinoma Cells *In Vivo*

EC cells express either a neoplastic or a normal embryonic phenotype depending upon their immediate environment. In an extra-embryonic environment, EC cells develop into malignant teratocarcinomas that contain a disorderly variety of differentiated tissues, including trophoblast, various epithelia, muscle (smooth and cardiac), cartilage, bone, etc. (Kleinsmith and Pierce, 1964). Furthermore, teratocarcinomas are capable of causing the death of the host animal. In contrast, when placed in an embryonic environment, these formerly neoplastic cells may behave as normal embryonic cells, responding to developmental signals and contributing to the formation of normal tissues in the resultant chimaeric mouse (Illmensee and Mintz, 1976).

Using cells taken directly from a transplantable tumour, Brinster (1974) was the first to show that EC cells can participate in the formation of a chimaeric mouse. Technically, this was achieved utilising a blastocyst injection procedure (Gardner, 1968). In addition to somatic contributions, two *in vivo* EC cell lines, both of which had appreciable subpopulations of cells with a normal karyotype, have also colonised the germ cell lineage and produced functional gametes in a limited number of founder chimaeras (Mintz and Illmensee, 1975; Illmensee and Mintz, 1976; Cronmiller and Mintz, 1978). From a total of three mice with *in vivo* EC-derived germ cells, two were males from a chromosomally XY ascites line (OTT 6050; a day 6.5 *p.c.* embryo-derived 129/Sv-Sl^J teratoma) and one was a female from a chromosomally XX solid tumour line (LT-72484-395; a spontaneous ovarian teratoma from the LT/Sv strain).

Papaioannou and co-workers (1975; 1978) were the first to demonstrate the capacity of EC cells that had been cultured *in vitro* to differentiate into the somatic lineages of resultant chimaeras. The first demonstration of germline transmission from an *in vitro* EC cell line was achieved by Stewart and Mintz (1981; 1982) working with a karyotypically normal XX embryo-derived teratocarcinoma cell line designated METT-1 (Mintz and Cronmiller, 1981). The chimaera-forming efficiency of METT-1 was low (13%), typical of that of most EC cell lines (reviewed by Robertson and Bradley, 1986), and with only 10% of the chimaeras having gonadal contributions, as assayed by GPI isoenzyme separation and germline transmission (Stewart and Mintz, 1981; 1982). Furthermore, only approximately 6% of the germ cells transmitted EC cell genotype in the two female germline chimaeras produced in these studies.

With regard solely to somatic chimaerism, the chromosomal constitution of an EC cell line may not be a good indicator of the *in vivo* pluripotency of the cells (a better assessment would be obtained from the *in vitro* differentiative capacity of the EC cells). For instance, cells with gross alterations in chromosome number and structure have been reported still to participate in the formation of apparently normal somatic tissues (Illmensee and Croce, 1979). It would appear that the interaction with normal host embryonic cells rescues the aneuploid EC cells. In contrast, some apparently normal euploid EC cell lines, such as C145b, have failed to make contributions to foetal tissues in any of several hundred mice analysed (Papaioannou *et al.*, 1979).

Other EC cell lines (for example P19: Papaioannou and Rossant, 1983) result in chimaeras with tumours. In fact, very few of the EC cell lines that have been examined have not resulted in the production of at least some tumour-bearing chimaeras. As summarised by Robertson and Bradley (1986), 17% of all liveborn

EC cell-chimaeras contained tumours and in an analysis of midgestation foetuses, 57% were judged to be abnormal and would have presumably failed to survive to term. It has been suggested that some of these tumour-bearing chimaeras may have arisen from failure of some injected EC cells to integrate with the ICM of the host blastocyst and which instead, proliferated independently as EC cell colonies (Papaioannou and Rossant, 1983). Thus, the number of EC cells introduced into the host embryo may have some bearing on the likelihood of tumour formation (Martin, 1980).

With the majority of chimaeric mice that have been generated via blastocyst injection, there has typically been very sporadic chimaerism; with EC cells not being present in all internal organs or tissues, nor in equal proportions in each tissue (Dewey *et al.*, 1977). The approach of aggregating small clumps of EC cells with one or two cleavage-stage host embryos was utilised to increase the intermingling of the two cell types in the chimaeric embryo (Fujii and Martin, 1980). However, Fujii and Martin (1983) have shown there to be a correlation between increasingly abnormal development of midgestation chimaeric foetuses, with more extensive participation of EC cells when aggregated with embryos. In the case of euploid EC cell lines, this situation may be attributable to genetic defects that are not detectable cytologically (Hardy *et al.*, 1990) and may reflect limitations in the ability of EC cells to direct normal development without the presence of substantial numbers of host embryonic cells (Fujii and Martin, 1983).

1.2.9 Summary

Teratocarcinomas are malignant tumours that may either arise spontaneously (in the testes or ovaries of certain inbred mouse strains) or experimentally, by grafting early embryos to an extra-uterine site. Their undifferentiated stem cells, embryonal carcinoma (EC) cells, have been isolated into culture from all three of the above sources. This questions the lineage of EC cells, as apparently the same cell type can be isolated from both male primordial germ cells and from cells within the early embryo. However, EC cells do have morphological, biochemical and immunological properties in common with pluripotent embryonic cells (see section 1.4). In some cases, *in vitro* differentiation of EC cells closely parallels development of early embryogenesis and has been utilised as an *in vitro* model system (see section 1.5). The most compelling evidence for the similarity between tumour stem cells and normal embryonic cells has been the ability of cells from some EC cell lines to participate in normal development, giving rise to chimaeric mice with somatic (and sometimes germline) EC cell contributions. This relationship is illustrated in figure 1.1.

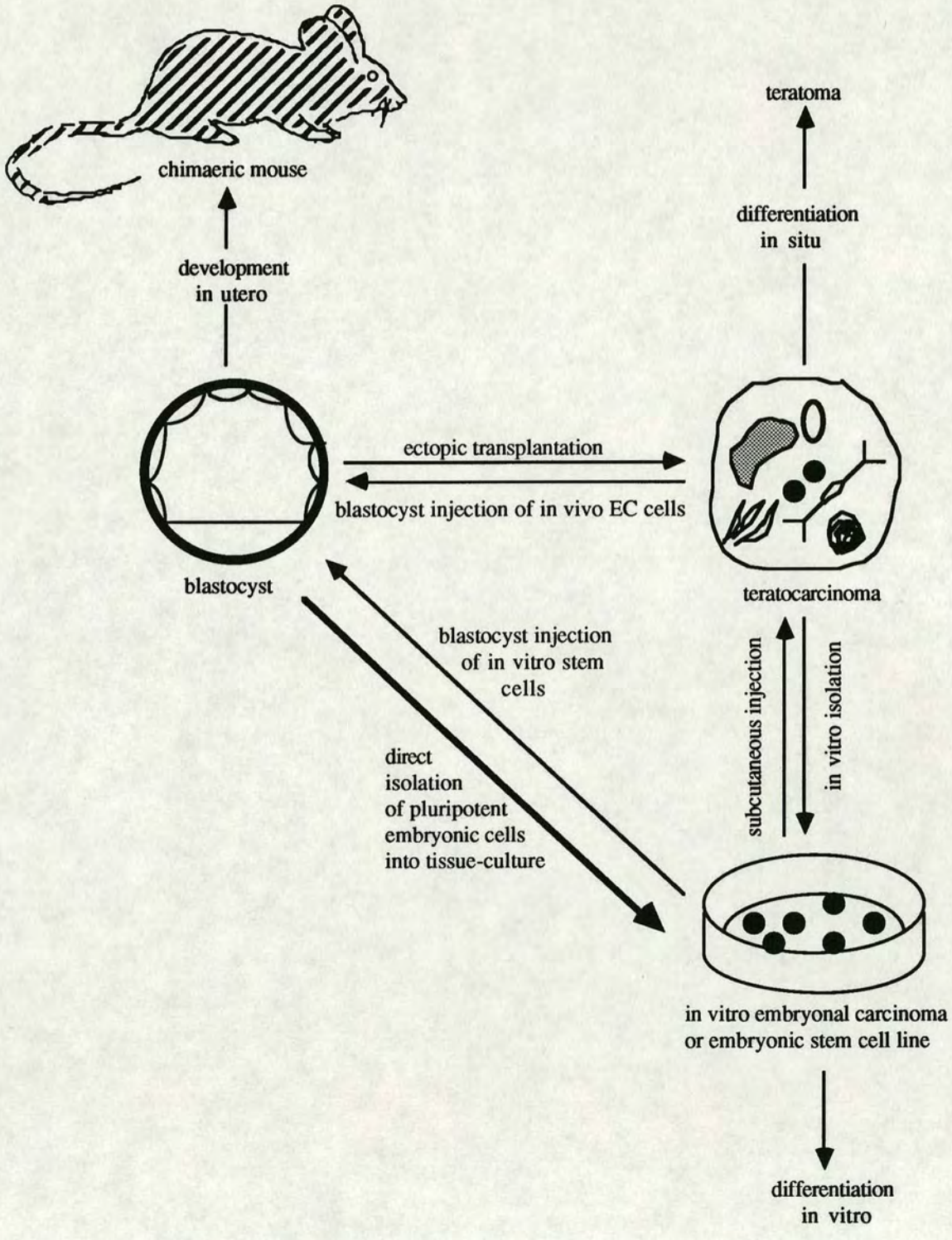


Figure 1.1: Relationship between normal murine embryos, embryonal carcinoma cells and embryonic stem cells. (Adapted from: Martin, 1981).

There is, however, large heterogeneity between the different EC cell lines with regard to karyotype, *in vitro* and *in vivo* differentiative capacities and in particular, germline transmission in chimaeras. This has limited their potential to create new mouse mutants via *in vitro* modification of the mouse genome (see section 1.5).

Many of the problems associated with EC cells may be attributed to the intermediate tumour phase in their isolation history. Thus, attempts were made in the late 1970's and early 1980's to isolate permanent cell cultures of undifferentiated morphology directly from the early embryo itself - as illustrated by the "thick" arrow in figure 1.1. The isolation of such "embryonic stem cell lines" forms the topic of section 1.3.

1.3 MURINE EMBRYONIC STEM CELL LINES

The isolation into tissue-culture of pluripotent cells directly from the embryo (by-passing teratocarcinoma formation; see figure 1.1) was first achieved by two independent laboratories, utilising different methods, in 1981 (Evans and Kaufman, 1981; Martin, 1981). These cell lines were initially termed EK cells by Evans and Kaufman, to distinguish them from the morphologically similar EC cells. Subsequently, however, they have been generally referred to as ES (embryonic stem) cells and this is the nomenclature used here. Under suitable culture conditions, ES cells are capable of continuous, undifferentiated growth *in vitro* (without cell transformation: Suda *et al.*, 1987). When re-introduced into an early embryo, ES cells are capable of differentiating in an organised manner and participating in normal embryo development (Bradley *et al.*, 1984).

This section reviews the early attempts to isolate cells of the ES cell lineage, followed by the eventual successful methods that were employed. Factors that may influence ES cell isolation and the characteristics of these stem cells are also reviewed.

1.3.1 Early Attempts on the Direct Isolation of Stem Cells into Culture

Evans (1981) reasoned that successful isolation may depend upon the identification of the developmental stage at which pluripotent cells within the embryo are capable of growth *in vitro* and explanting sufficient numbers of these precursor cells into a culture environment conducive to proliferation, as opposed to differentiation. Initial attempts at direct isolation of pluripotent cells were made from both early embryos and primordial germ cells, since studies on teratocarcinomas had

shown that cells with a stem cell morphology can be isolated from both of these sources (see section 1.2).

The explanted germinal ridge has not, however, been a viable route to the isolation of stem cells, as primordial germ cells from day 11.5 or 12.5 *p.c.* germinal ridges failed to divide in culture despite variations in media and feeder cells (Evans, 1981). Transient proliferation has been achieved by decreasing the culture temperature to below 30°C, however, the primordial germ cells senesced within seven days (De Felici and McLaren, 1983). In contrast, the early embryo has provided a more amenable source of cells for *in vitro* growth.

Cell surface antigens and patterns of protein synthesis within the early embryo have revealed that neither the cells of the day 6.5 *p.c.* ectoderm nor those of the day 3.5 *p.c.* ICM share homology with EC cells, but cells of the day 5.5 *p.c.* primitive ectoderm may do so (Martin, Smith and Epstein, 1978; Evans *et al.*, 1979; Lovell-Badge and Evans, 1980). Thus, cells from the early post-implantation embryo seemed the best candidates for progenitors of pluripotent cells in culture. Certainly, explant cultures from more advanced day 6.5 and 7.5 *p.c.* embryos only exhibited extensive differentiation (Evans, 1981). However, because of technical difficulties in isolating the day 5.5 *p.c.* primitive ectoderm, early efforts concentrated instead on culturing day 3.5 *p.c.* intact blastocysts (also capable of forming teratocarcinomas; see section 1.2.3) or isolated ICMs, following immunosurgery (Solter and Knowles, 1975). Initial attempts to culture embryos in a variety of different media, were unsuccessful in establishing progressively growing cultures of pluripotent cells (Sherman, 1975a;b). However, differentiated cell lines of fibroblastoid and endodermal-like morphology were established from mouse embryos (Sherman, 1975b) although cells possessing an undifferentiated phenotype had been transiently observed in culture in some early studies (Solter and Knowles, 1975; Atienza-Samols and Sherman, 1978).

1.3.2 Establishment of Pluripotent Cells from Early Mouse Embryos

The direct isolation of stable cell lines with an undifferentiated morphology from mouse embryos was first achieved by Evans and Kaufman (1981) and followed shortly after by Martin (1981). They used quite different strategies, but ultimately achieved success by diverting the ICM from its normal fate of differentiation and instead encouraging continued proliferation *in vitro*.

Evans and Kaufman (1981) used treatments (ovariectomy and progesterone injection) to induce mouse blastocysts to undergo a short period of

implantational delay; whereby the embryos hatched from the zona pellucida but were allowed to remain free-floating within the uterine lumen for three to five days before embryo recovery. Such a treatment has been shown to produce a small increase in the cell number of the ICM (Copp, 1982) and although primitive endoderm may differentiate, no further development of the ICM occurs (Gardner, Davis and Carey, 1988). And so, the embryo enters a state of diapause.

These delayed blastocysts were group-cultured in microdrops of tissue-culture medium, under paraffin oil on tissue-culture plastic Petri dishes (Evans and Kaufman, 1981). The intact blastocysts attempt to mimic implantation via attachment of the trophectodermal cells to the culture surface. The cells then grow out as a monolayer and differentiate into giant trophoblast cells. This abnormal *in vitro* development, results in exposure of the ICM to the culture environment. The ICM subsequently develops into an upwardly growing egg cylinder-like structure after about four days. Earlier studies had shown that such outgrowths only gave differentiated cell phenotypes if left unmanipulated (Sherman, 1975b). Thus, Evans and Kaufman (1981) disaggregated the ICM outgrowths using enzymatic and mechanical procedures. The embryonic cells were then passaged onto gelatin-pretreated culture dishes containing a feeder cell layer of mitotically inactivated mouse STO fibroblasts (Martin and Evans, 1975). Cell colonies of EC-like morphology were identified in these primary cultures and were subsequently picked out, passaged and mass cultures grown (Evans and Kaufman, 1981). In one experiment, 30% of the delayed blastocysts explanted into culture gave rise to permanent stem cell lines. Cultures were routinely passaged by trypsinisation every two to three days and co-cultured on STO feeder cells to maintain the ES cells in an undifferentiated state. The capacity of the ES cells to differentiate *in vitro* (forming embryoid bodies) and *in vivo* (forming teratocarcinomas) demonstrated that these cells were pluripotent (Evans and Kaufman, 1981).

With a different experimental technique, Martin (1981) described the isolation of ES cells which were of the same lineage as the cells isolated by Evans and Kaufman (1981). Martin established her ES cell lines from immunosurgically isolated ICMs from day 3.5 *p.c.* blastocysts which were cultured together on STO feeder cells in medium that had been conditioned by an undifferentiated EC cell line (PSA-1). A proportion of such ICMs (4/30) were reported to give rise directly to colonies of stem cells, apparently without the necessity of any further specific manipulation. The failure of control ICMs, cultured in the absence of conditioned medium, to give rise to ES cells suggested the production of a factor(s) by the PSA-1 EC cell line that either stimulated the proliferation or inhibited the differentiation of stem cells, or both. After five culture passages, conditioned medium was not required for the continued, stable

growth of the stem cell phenotype. This suggested the production of an autostimulatory growth factor(s) by these ES cells. Martin (1981) also demonstrated that each ES cell was pluripotent, as subclonal lines produced teratocarcinomas following injection into athymic mice.

In a comparative study, Axelrod and Lader (1983) showed that the efficiency of ES cell isolation from ICMs cultured in either conditioned medium or normal medium were similar (2/17 and 2/16, respectively). They also demonstrated that ES cell lines could be derived from the outgrowths of intact day 3.5 *p.c.* blastocysts (at an efficiency of 6%) without the need to induce implantational delay. Hence, they demonstrated the feasibility of simplified techniques for the establishment of ES cell lines without the need for the production of conditioned medium, immunosurgery or ovariectomy.

1.3.3 Factors Influencing the Isolation of Murine Embryonic Stem Cells

There are few comparative reports regarding factors that influence the efficiency of ES cell isolation from mouse embryos. Despite differences which exist between some laboratories, this section reviews a number of important variables.

1.3.3.1 Disaggregation of ICM Outgrowths

In terms of manipulating the embryo to achieve optimal proliferation of subsequent primary stem cell colonies, the timing of the disaggregation of the ICM outgrowth is of critical importance. In a detailed account of the technique, Robertson (1987) has found that day 3.5 *p.c.* blastocysts usually attain a suitable morphology after a five day culture period, although variability exists between embryos in the rate of growth *in vitro*. Allowances may have to made for embryos recovered following a period of implantational delay, which tend to show slower rates of *in vitro* growth initially (Robertson and Bradley, 1986). Based on her experience, Robertson (1987) has noted that there was no absolute correlation between the phenotype of the ICM outgrowth and the successful isolation of an ES cell line. However, outgrowths with extensive endoderm formation or those developing into multi-layered egg cylinder-like structures, have a reduced capacity to retain pluripotent cells.

Another important factor is the disaggregation procedure itself. Cell survival is poor if the ICM outgrowth is trypsinised into single-cells (Robertson,

1987). The aim should be to break the ICM up into several small pieces, each containing a few cells.

1.3.3.2 Mouse Strain

One of the restrictions of EC cells is that they may only be isolated from teratocarcinomas derived from inbred lines of mice (see section 1.2.3). In contrast, ES cell lines have been isolated from inbred strains (*e.g.* 129/Sv//Ev: Evans and Kaufman, 1981; C57BL/6: Suemori and Nakatsuji, 1987) outbred strains (*e.g.* CD-1: Suda *et al.*, 1987; CFLP: Robertson *et al.*, 1983b) and numerous different F_1 crosses (*e.g.* ICR X SWR/J: Martin, 1981; C57BL/6 X C3H: Martin and Lock, 1983). Furthermore, ES cell lines have been produced which specifically carry a variety of genetic markers. These include lines carrying isozymal variants (GPI-1^a: Bradley *et al.*, 1984), lines with an isozymally marked X chromosome (*Pgk-1*^{a}: Martin and Lock, 1983), lines with prominent translocation markers (Rb[16·17]: Axelrod and Lader, 1983) and lines carrying a range of coat-colour phenotypes (Robertson *et al.*, 1983b). A valuable use of this technique has been to produce ES cell lines from homozygous recessive, developmentally lethal mutations (*e.g.* t^{w5}/t^{w5}: Magnuson *et al.*, 1982; 1983).

In a laboratory experienced in ES cell isolation, Robertson and Bradley (1986) have not found any specific mouse strain to be "non-permissive" and routinely expect 10% of day 3.5 *p.c.* blastocysts to yield ES cell lines. Also, in a study primarily examining the effect of feeder cell layers, there were no significant differences between the embryos from the inbred C57BL/6 and 129 (/J and /SvJ) strains and the F_1 (C57BL/6 X 129) hybrid embryos, in the efficiency of ES cell isolation (18%, 18% and 12%, respectively; Suemori and Nakatuji, 1987). In contrast, the embryos from some mouse strains have been observed to yield ES cell lines at a lower frequency (5-10%) compared to others (35-45%), although no specific mouse strains were mentioned (Martin, Jakobovits and Joyner, 1984).

1.3.3.3 Stage of Embryonic Development

ES cells have been isolated from the blastomeres of day 2.5 *p.c.* morulae and from the ICM of blastocyst-stage pre-implantation mouse embryos. ES cell lines derived from the ICM have been obtained from fertilised day 3.5 *p.c.* or implantationally delayed blastocysts and from parthenogenetically and androgenetically produced blastocyst embryos.

The majority of workers have isolated ES cell lines from the readily available day 3.5 *p.c.* blastocyst-stage embryo. In a small study comparing isolation from either intact blastocysts or isolated ICMs from day 3.5 *p.c.* embryos, Axelrod and Lader (1983) found there to be no difference in the frequency of ES cell isolation (2/23=9% *vs* 2/16=13%, respectively). From experience, Robertson and Bradley (1986) expect blastocysts that have undergone a period of implantational delay to generate, on average, a three-fold increase in the efficiency of ES cell isolation compared to non-delayed, day 3.5 *p.c.* embryos.

Given that ES cell isolation from both non-delayed and delayed blastocysts relies on the proliferation of the ICM, the only other normal developmental stage from which stem cells have been obtained is from disaggregated 16- to 20-cell embryos (Eistetter, 1989). Here, individual blastomeres from decompacted morulae were cultured and 37% (17/46) of the initial embryos produced permanent ES cell lines with, in some instances, as many as four blastomeres from a single embryo giving rise to stem cell colonies. This was a significantly greater efficiency of ES cell isolation than in control experiments, where blastocysts were cultured conventionally (9/108=8%; Eistetter, 1989). The blastomere-derived cell lines appear to be of the same lineage as ICM-derived ES cells, although a chimaeric analysis has not been reported.

Several ES cell lines have been isolated from blastocyst outgrowths derived from parthenogenetically activated oocytes (Kaufman *et al.*, 1983). Both haploid and diploid parthenogenetic blastocysts gave rise to **diploid** ES cell lines, which have been "rescued" *in vivo*, to contribute to adult somatic chimaeras (Evans, Bradley and Robertson, 1985).

Diploid androgenetic ES cells have also been produced (Mann *et al.*, 1990). The *in vivo* differentiation of these androgenetically-derived stem cells into teratomas composed predominantly of muscle, and the postnatal skeletal abnormalities in chimaeras, suggests stage- and tissue-specific expression of developmentally important imprinted genes (Mann *et al.*, 1990).

1.3.3.4 Feeder Cells

Historically, ES cells have been isolated and maintained on layers of mitotically inactivated, embryonic fibroblast "feeder" cells. If cultured in the absence of feeders, the ES cells rapidly differentiate (Evans and Kaufman, 1981). The function of the feeder cells, in addition to providing a more suitable attachment surface for direct co-culture (Martin and Evans, 1975), is in the active suppression of stem cell differentiation. It has recently been shown that a factor known as DIA/LIF

(see section 1.3.3.5), which inhibits the differentiation of stem cells, is produced by these feeder cells as both a diffusible protein and in an immobilised form, associated with the extra-cellular matrix (Rathjen *et al.*, 1990b). Furthermore, it has been shown that in the co-culture system, ES cells secrete a heparin-binding growth factor responsible for the stimulation of DIA/LIF expression in the feeder cells (Rathjen *et al.*, 1990a).

The most commonly utilised fibroblast feeder layers have been those prepared from the STO cell line (Martin and Evans, 1975); a thioguanine- and ouabain-resistant derivative of a continuous embryonic fibroblast cell line isolated from the SIM mouse strain (Ware and Axelrad, 1972). Other embryonic fibroblast cell lines also capable of maintaining stem cells include C3H 10T1/2 cells (Rathjen *et al.*, 1990b) and BALB-3T3/A31 cells (Ogiso *et al.*, 1982). Alternatively, primary embryonic fibroblasts may be isolated from foetuses in the third trimester of pregnancy. Although such feeders have been favoured by some workers (Doetschman *et al.*, 1985) a major disadvantage in their use is the limited *in vitro* life-span of these primary cells; they become senescent after 15-20 cell divisions (Robertson, 1987). In order to prepare feeder cell layers for co-culture, the fibroblast cells must be mitotically inactivated. Typically, this has been achieved by treatment with the drug mitomycin C (Martin and Evans, 1975) or by exposure to irradiation (Wobus *et al.*, 1984).

In a comparative study, there was no significant difference in the efficiency of ES cell isolation between STO and primary embryonic fibroblast feeder cell layers (62/361=17% and 24/175=14%, respectively; Suemori and Nakatsuji, 1987). However, primary fibroblasts were superior in supporting other stem cell characteristics. ES cells grown on the STO feeders tended to be morphologically unstable, which was reflected in their capacity to form only differentiated teratomas *in vivo*. Furthermore, ES cell lines isolated on the STOs became tetraploid, whereas those maintained on the primary fibroblasts were karyotypically stable. The above results are in conflict with the findings of other laboratories which routinely use STO feeders, where the majority (77%) of ES cell lines have been reported to be euploid (Robertson and Bradley, 1986). This may emphasise differences in the culture conditions between different laboratories. Also, it would appear that ES cells adapt to the culture environment they are grown in, as an ES cell line originally isolated on primary embryonic fibroblasts could not be maintained subsequently on STO feeders (Wobus *et al.*, 1984).

The effect of the feeder cell layer can be fully substituted by the addition of an exogenous factor (DIA/LIF) into the medium, which maintains the ES cell morphology of established lines and is also sufficient for the isolation of new stem

cell lines. The effects of cell-conditioned medium and DIA/LIF form the subject of the following section.

1.3.3.5 Cell-Conditioned Medium and Differentiation Inhibiting Factors

The tissue-culture medium utilised is based upon a complex bicarbonate-buffered formulation, typically Dulbecco's Modification of Eagle's Medium (DMEM; Robertson, 1987) or Glasgow's MEM (Handyside *et al.*, 1989). To this is added ß-mercaptoethanol (which enhances stem cell attachment and growth; Oshima, 1978) and non-essential amino acids (supplemented to prevent cellular loss across concentration gradients; Hooper, 1987). For ES cell isolation, serum concentrations between 10% (Martin, 1981) and 20% (Evans and Kaufman, 1981) have been successfully employed. Typically, serum has been a 50:50 combination of (selected batches) of foetal and newborn calf serum. Once ES cell lines have been established, the serum content may be reduced for routine maintenance *in vitro* (Robertson, 1987).

Martin (1981) first demonstrated the use of cell-conditioned medium to facilitate ES cell isolation. The PSA-1 EC cell line used to produce this conditioned medium, was subsequently shown to secrete a factor (SCGF-1) which stimulated the proliferation of the stem cells that produced it (Jakobovits, Banda and Martin, 1985). Another potent mitogen, ECDGF has also been isolated from medium conditioned on the PC13 EC cell line (Heath and Smith, 1988).

As all ES cell lines are feeder-dependent, with feeder cell removal resulting in ES cell differentiation, this suggested the presence of a differentiation inhibitory factor produced by the feeder cells. Medium conditioned on STO feeder cells (Smith and Hooper, 1983) and on a variety of other cell types from different species (Koopman and Cotton, 1984) were shown to only partially inhibit stem cell differentiation when cultured without feeders. It has since been shown that the major effect of the feeder layer is in the synthesis of an immobilised anti-differentiation factor, associated with the extra-cellular matrix and not only from the production of a diffusible factor (Rathjen *et al.*, 1990b).

Smith and Hooper (1987) found that medium conditioned by Buffalo rat liver (BRL) cells (Coon, 1968) contained a factor which was a far more potent inhibitor of stem cell differentiation than medium conditioned by STO cells. This factor, termed stem cell differentiation inhibiting activity (DIA), has been purified from BRL-conditioned medium and is a glycoprotein of M_r 43 000 (Smith *et al.*, 1988). Structural and functional comparisons have shown that DIA is identical to the

murine myeloid leukemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988). Furthermore, a third growth factor, human interleukin for DA cells (HILDA), has been shown to be essentially identical to the human LIF protein (Moreau *et al.*, 1988; Gough *et al.*, 1988).

The supplementation of DIA/LIF to medium has provided a complete substitute for feeder cells and hence, has greatly simplified the routine culture of ES cell lines. In addition, DIA/LIF has aided the biochemical study and *in vitro* manipulation of ES cells. Established ES cells have retained their full developmental potential and capacity to colonise the germline in chimaeras when maintained in the "crude" BRL-conditioned medium (Hooper *et al.*, 1987) and in the presence of purified recombinant DIA/LIF (Gough *et al.*, 1989).

The isolation of ES cells has been aided by the combined use of BRL-conditioned medium and STO fibroblasts (Handyside *et al.*, 1989). However, Robertson and Bradley (1986) have observed that while conditioned medium may enhance the growth of primary colonies, the majority tend to be of a "pre-trophoblast" lineage and ultimately differentiate. It has recently been shown that recombinant DIA/LIF, added in the culture medium, can replace feeder cells in the isolation of ES cell lines, which may retain their capacity for germline transmission (Nichols, Evans and Smith, 1990; Pease *et al.*, 1990).

DIA/LIF now appears to be implicated as a component governing stem cell proliferation and differentiation during mammalian development; for it has bioregulatory roles not only in the early embryo (Conquet and Brulet, 1990; Rathjen *et al.*, 1990a) but also in other systems, including haematopoietic (Leary *et al.*, 1990), leukaemic (Hilton *et al.*, 1988) and osteogenic lineages (Abe *et al.*, 1986; Metcalf and Gearing, 1989).

1.3.4 Differentiation of Embryonic Stem Cells *In Vitro*

A universal feature of ES cell lines appears to be their capacity for spontaneous differentiation under suspension culture; forming three-dimensional structures known as embryoid bodies (Evans and Kaufman, 1981; Martin, 1981). With prolonged culture, 50% of these embryoid bodies, from a particular cell line, may expand into large cystic structures containing alphafoetoprotein and transferrin, thus being analogous to the visceral yolk sac of the post-implantation embryo (Doetschman *et al.*, 1985). A proportion of the cystic embryoid bodies may develop beating myocardium, and when cultured in human cord serum, blood islands also form (Doetschman *et al.*, 1985). More extensive haematopoietic differentiation has been induced by culturing embryoid bodies in semi-solid medium (Wiles and Keller,

1991). Attachment of embryoid bodies to a culture surface may result in the growth of an array of cell types, including: nerve, adipose, muscle, cartilage and sometimes trophectoderm (Martin, 1981; Axelrod and Lader, 1983).

ES cells can also be induced to differentiate in monolayer culture. When grown in DIA/LIF, the addition of retinoic acid induces parietal endoderm formation, while removal of DIA/LIF from the medium results in a population of ill-defined mesoderm-like cells (Mummery *et al.*, 1990).

1.3.5 Chromosomal Characteristics of Embryonic Stem Cells

The majority of ES cell lines, at least initially, have a normal euploid chromosome complement (Robertson and Bradley, 1986). This may be a result of the rapid isolation process, especially compared to tumour-derived EC cells. However, within most ES cell lines small populations of aneuploid cells may exist. With increasing time *in vitro*, the proportion of these pseudodiploid cells may increase (Suda *et al.*, 1987). To regenerate a karyotypically normal cell line, the ES cells may be single-cell cloned and diploid cultures identified and re-expanded (Suda *et al.*, 1987).

Established ES cell lines cultured without feeders, but in the presence of BRL-conditioned medium or DIA/LIF-containing medium, presumably retain a normal karyotype, based on the capacity of the stem cells to enter the germline in chimaeric mice (Handyside *et al.*, 1989; Hooper *et al.*, 1987; Gough *et al.*, 1989). In terms of isolating karyotypically normal cell lines, however, co-culture with feeder cells may be important, as 93% of the ES cell lines established solely in DIA/LIF-containing medium have been reported to possess significant proportions of cells with chromosomal abnormalities (*cf.* Nichols *et al.*, 1990 and Robertson and Bradley, 1986).

A common karyotypic abnormality found in the autosomal chromosome complement of ES cells has been trisomy 11 (Robertson and Bradley, 1986; Crolla, Brown and Whittingham, 1990). Chromosome 11 has also been found to be unstable in EC cells (McBurney, 1976).

It has been noted in one laboratory that the majority (25/35) of ES cell lines from fertilised embryos have been male (XY) (Robertson and Bradley, 1986). Only five lines were female (XX) with the remainder being XO. This might suggest that undifferentiated cells possessing two active X chromosomes were discriminated against during the isolation process and the loss of one X chromosome may confer some selective growth advantage (Evans and Kaufman, 1983). Interestingly, all of the female ES cell lines isolated in DIA/LIF and without feeder cells, were either

wholly or partially tetraploid (Nichols *et al.*, 1990). Parthenogenetically-derived ES cell lines have two (maternally) active X chromosomes (Rastan and Robertson, 1985) and the occurrence of a partial deletion of one X chromosome in these cell lines has been suggested to represent a compensatory mechanism for X inactivation (Robertson, Evans and Kaufman, 1983a). The extent of this deletion was variable between individual parthenogenetically-derived ES cell lines, however, the break-point on the X chromosome appeared to be constant within a particular cell line (Robertson *et al.*, 1983a). The partial deletion of a single X chromosome has only been reported for one ES cell line derived from a fertilised embryo (Martin, 1981).

1.3.6 Differentiation of Embryonic Stem Cells *In Vivo*

ES cells have been demonstrated to be pluripotent from the capacity to form teratocarcinomas (Evans and Kaufman, 1981; Martin, 1981) and germline chimaeric mice (Bradley *et al.*, 1984), dependent upon the environment into which the stem cells are introduced.

A study within one laboratory has found that without exception, all 17 independently derived ES cell lines tested were capable of producing normal chimaeric mice following blastocyst injection (Evans *et al.*, 1985). This seems to be true for a number of different laboratories (reviewed by Baribault and Kemler, 1989) and is in contrast to EC cells (see section 1.2.8). Typically, ES-chimaeras have been produced at high efficiency (35%), with the stem cells often contributing to 50% of all somatic tissues within the foetus or adult (Evans *et al.*, 1985). Occasionally, trophectoderm and primitive endoderm derivatives in the extra-embryonic tissues, may also be colonised by ES cells (Beddington and Robertson, 1989; Suemori *et al.*, 1990). Evans and associates (1985) found no significant foetal losses, nor any evidence of tumour formation in any of the 420 chimaeras produced. In fact, there has been only one report of an extra-gonadal teratocarcinoma derived from ES cells in a chimaeric mouse; presumably resulting as a consequence of the "escape" of one or more stem cells from the regulative control of the ICM, following injection into the host blastocyst (Hardy *et al.*, 1990).

Using an euploid XY ES cell line, Bradley and colleagues (1984) first demonstrated the capacity of ES cells to colonise the germline in 19% of chimaeric males set-up for test breeding. In the chimaeric population, they noted a marked sex-distortion effect; with 70% of chimaeras being phenotypic males. Because the Y chromosome is principally responsible for sex determination in mammals, injection of XY stem cells into an XX blastocyst, results in sex-conversion in a proportion of formerly female embryos. This is a localised effect, dependent upon the relative

contribution of host XX and ES-derived XY somatic cells in the germinal ridge (reviewed by Robertson, 1986). A small XY contribution may only exert a masculinising influence and result in animals which, although phenotypically male, may be sterile hermaphrodites. This may occur in two-thirds of the sexually converted chimaeras produced from the XY stem cell - XX embryo combination (Robertson and Bradley, 1986). The remaining fertile male, sex-converted animals transmitted **only** the XY ES cell-derived genotype to their progeny (Bradley *et al.*, 1984). Although these animals were probably sexual mosaics, XX host germ cells appear incapable of forming functional spermatozoa (McLaren, 1976).

In an alternative sex combination (XY ES cells injected into an XY embryo) the phenotypic male chimaeras produced may transmit the ES genotype in only 0.3 to 3% of their sperm (Robertson, 1986).

In achieving germline chimaerism, male ES cell lines have a greater potential, because the XY constitution is more stable *in vitro* (see section 1.3.5), sex-conversion produces some chimaeric males that transmit only the ES cell genotype and breeding from males is more rapid. There are no reports of germline chimaeras from XX ES cell lines; probably because this event has not been extensively assayed. However, *in vitro* XX EC cells have colonised the female germline (see section 1.2.8) and XO ES cells have given rise to germline female chimaeras (Kuehn *et al.*, 1987). Apart from perhaps the loss of one sex chromosome, a normal, stable autosomal chromosome complement is apparently vital for transmission through the gametes. This generally implies the use of ES cells with a short culture history. However, germline transmission from ES cells following 260 cell generations *in vitro* has been demonstrated; but not without periodic subcloning (Suda *et al.*, 1987).

It is worth noting that the vast majority of workers that have demonstrated germline transmission from ES-chimaeras, have utilised ES cells derived from embryos of the 129 mouse strain (including a number of sub-strains; for a review see Baribault and Kemler, 1989). The only other mouse strain reported in the literature, from which ES cells have been derived and have produced germline chimaeras, is the CD-1 strain (Suda *et al.*, 1987). However, there is no biological reason to assume that euploid ES cells derived from the embryos of any mouse strain would not be capable of colonising the germline.

Factors which may increase the contribution of ES cells into the germline of chimaeras include: **1.)** increasing the number of stem cells injected (from three or five to 10-15 cells: Robertson and Bradley, 1986); **2.)** the choice of mouse strain from which to obtain host blastocysts (*e.g.* stem cells have entered the germline at a greater frequency when injected into C57BL/6 host embryos compared to MF1 or CD-1 strain embryos: Schwartzberg, Goff and Robertson, 1989); **3.)** utilising strains

of mice carrying specific fertility mutations to produce host blastocysts (*e.g.* the *W* mutation ("dominant white spotting"): Robertson and Bradley, 1986); **4.)** and the developmental stage of the host embryo (Lallemand and Brulet, 1990). The injection of ES cells into morula-stage embryos has shown more extensive chimaerism in midgestation foetuses compared to those from blastocyst injection (Lallemand and Brulet, 1990). This may in turn increase the probability of obtaining germline chimaeras and appears more efficient than earlier attempts at ES cell-morula aggregation experiments (Stewart, Vanek and Wagner, 1985).

Recent studies have shown murine ES cells to be capable of supporting complete foetal development (Nagy *et al.*, 1990). Newborn pups were produced which were 100% ES cell-derived, following the aggregation of stem cells with tetraploid cleavage-stage embryos. Tetraploid embryos were used in order to establish the placental connection (Nagy *et al.*, 1990), as ES cells are not capable of contributing extensively to these extra-embryonic tissues (Beddington and Robertson, 1989). The reason why these ES-derived newborn pups failed to survive after birth is not yet clear (Nagy *et al.*, 1990).

1.3.7 Summary

Murine embryonic stem cells (ES cells) are immortal, pluripotent cell lines derived directly from the pre-implantation embryo. Cultured in the presence of either feeder cells or in medium containing DIA/LIF, ES cells maintain their undifferentiated phenotype. However, following introduction into early host embryos and transfer to the uteri of pseudopregnant female recipients, ES cells respond to normal developmental signals and are capable of contributing to the full range of foetal tissues, resulting in the formation of chimaeric offspring. Mosaicism may extend into the germ cell lineage and ES cells may contribute fully functional gametes. Consequently, ES cells are being increasingly utilised as cellular vectors for genetic modifications of the mouse genome (see section 1.5). Because ES cells can theoretically be isolated from any mouse strain, cell lines can be designed to carry the desired genetic background.

ES cells represent a relatively homogeneous set of cell lines with respect to *in vitro* and *in vivo* differentiation characteristics. Generally, ES cells are karyotypically stable, at least during early cell generations. This is attributable to the rapid and direct nature of their isolation from the embryo. The exact relationship between EC cells, ES cells and the pluripotent cells within the mouse embryo, is reviewed in section 1.4.

1.4 RELATIONSHIP BETWEEN CULTURED PLURIPOTENT STEM CELLS AND CELLS IN THE EARLY MURINE EMBRYO

The basic difference between cultured stem cells and pluripotent cells within the early mouse embryo is the capacity of the cultured cells for apparently unlimited proliferation, whilst still retaining their pluripotency *in vivo* (Suda *et al.*, 1987). In contrast, during normal embryonic development there is a continuous restriction, albeit gradual, in the potency of the cells as differentiation proceeds (Gardner and Beddington, 1988). This section reviews the embryonic lineage that EC and ES cells best represent in the normal embryo and considers the two hypotheses put forward to explain how early embryonic cells can be isolated *in vitro* and diverted from their normal fate of differentiation. That is, are pluripotent stem cells normal embryonic cells, or is an epigenetic modification necessary for the isolation of stem cells into culture ?

1.4.1 The Stem Cell Lineage

Within the embryo a population of pluripotent cells exists up to day 7.5 *p.c.* of development; with the primitive ectoderm subsequently undergoing gastrulation, forming the definitive embryonic ectoderm, endoderm and mesoderm layers of the foetus (see section 1.1). Other embryonic cells that may be considered pluripotent are the progenitors of the germ cell lineage. Both male primordial germ cells and early embryos (up to day 7.5 *p.c.*) are capable of forming teratocarcinomas from which EC cells may be isolated (see section 1.2). EC cells from both of these sources appear morphologically and functionally similar (Martin, 1980; Evans, 1981) and there are great similarities in the route of tumour formation. Spontaneous activation of oocytes (Stevens and Varnum, 1974) and ectopic transfer of pre-implantation embryos (Stevens, 1968) may result in development of normal egg cylinder-stage embryos before they become disorganised in the foreign environment and lead to a tumourous growth. Interestingly, in the initial phase of tumour induction, undifferentiated primordial germ cells also proliferate into cells resembling primitive embryonic ectoderm (Stevens, 1983) and it is this tissue within the egg cylinder-stage embryo which is responsible for teratocarcinoma formation (Diwan and Stevens, 1976). Thus, there appears to be a common stage after which there may be malignant conversion. For the optimal isolation of ES cells from embryonic outgrowths of intact blastocysts, the ICM has also been been allowed to develop into structures resembling early egg cylinder-stage embryos, before disaggregation *in vitro* (Robertson, 1987). However, this is not essential in view of the direct establishment

of ES cells from isolated ICMs (Martin, 1981) and blastomeres, from disaggregated morulae (Eistetter, 1989).

In addition to morphological similarities between primitive ectoderm, ES cells, EC cells (Evans and Kaufman, 1981; Martin, 1981) and primordial germ cells (Pierce and Beals, 1964), there are biochemical and immunocytochemical similarities also. The cell surface antigen expression of SSEA-1 appears to be correlated with developmental pluripotency; first appearing on the blastomeres of eight-cell embryos (Solter and Knowles, 1978), then subsequently becoming restricted to the ICM, primitive ectoderm, primordial germ cells, EC cells (Fox *et al.*, 1981) and ES cells (Martin and Lock, 1983). Pluripotent stem cells fail to express cytoskeletal markers indicative of differentiated cell types (Mummery *et al.*, 1990). High alkaline phosphatase enzyme activities have been observed in primordial germ cells (Ginsburg *et al.*, 1990), EC cells (Bernstine *et al.*, 1973) and ES cells (Wobus *et al.*, 1984). Patterns of protein synthesis from EC cells and ES cells have shown greatest homology with the day 5.5 *p.c.* primitive ectoderm (Martin, Smith and Epstein, 1978; Evans *et al.*, 1979; Lovell-Badge and Evans, 1980; Evans and Kaufman, 1983). However, detailed *in vivo* studies utilising ES cells have shown that these stem cells do not share the restricted developmental potential of primitive ectoderm; which by day 4.5 *p.c.* is incapable of contributing to primitive endoderm and trophectodermal lineages (Gardner and Rossant, 1979; Gardner, 1985). ES cells on the other hand, have been shown to colonise these tissues in chimaeras, albeit at low frequency (Beddington and Robertson, 1989; Suemori *et al.*, 1990). In this respect, ES cells resemble early ICM cells in their developmental potency (Rossant and Lis, 1979; Gardner, 1985). This was all the more noteworthy as the cell lines utilised by Beddington and Robertson (1989) were derived from implantationally delayed blastocysts, in which the ICMs would have already differentiated into primitive ectoderm and primitive endoderm (Gardner *et al.*, 1988). Thus, there may be some "de-differentiation" within the ES cell lineage. It would be interesting to conduct a mid-gestational chimaeric analysis comparing the colonisation of ES cell lines derived from the blastomeres of morula-stage embryos (Eistetter, 1989) with those derived from blastocysts.

It has been difficult to assess subtle differences between EC cell lines, derived from different stage embryos or primordial germ cells, in the colonisation of extra-embryonic membranes in chimaeras because of the great heterogeneity that exists between the cell lines in general. Although, as suggested earlier, teratocarcinomas may develop through a common cell type (the primitive ectoderm) some stage differences in developmental potency, similar to the normal embryo, have been reported. Re-transplantable teratomas containing trophoblast tissue have been

produced following the injection of single EC cells derived from primary testicular teratocarcinomas (Kleinsmith and Pierce, 1964) and teratocarcinomas derived from day 3.5 *p.c.* embryos (Evans, 1972) and hence, are of a similar developmental potential to immunosurgically isolated, early ICMs (Rossant and Lis, 1979). However, trophoblast was apparently not amongst the tissues present in teratomas produced from EC cells derived from a day 6.5 *p.c.* embryo (Mintz and Cronmiller, 1981). Primitive endoderm derivatives have been observed in differentiated cell cultures and in chimaeric mice, with EC cells derived from day 3.5 *p.c.* embryos (Martin and Evans, 1975; Stewart, 1982), but not with EC cells derived from some day 7.5 *p.c.* embryos (Rossant and Papaioannou, 1984).

Differences with regard to X chromosome inactivation have also been reported with stem cells derived from different embryonic stages. Within the primitive ectoderm of female embryos, random inactivation of one X chromosome is completed by day 5.5 *p.c.* (Rastan, 1982). With ES cells derived from the ICM of parthenogenetically activated embryos, both X chromosomes are active (Rastan and Robertson, 1985). Some XX EC cell lines derived from older day 7.5 *p.c.* embryos have undergone X-inactivation, as would be expected *in vivo* (McBurney and Adamson, 1976). However, other EC cell lines derived from day 7.5 *p.c.* embryos have two active X chromosomes, thought to arise as a result of reactivation of a previously inactive X chromosome during the process of teratocarcinogenesis (McBurney and Strutt, 1980). Differentiation of these EC cells *in vitro* was accompanied by the inactivation of one X chromosome (McBurney and Strutt, 1980).

Because of the ability to isolate EC cells from both primordial germ cells and early embryos, it was initially thought that EC cells derived from embryos may have originated from those cells of the primitive ectoderm that would form the germ cell lineage (Papaioannou, Rossant and Gardner, 1978). The earliest sighting of primordial germ cells has not been before day 7.5 *p.c.* (Ginsburg *et al.*, 1990), however, it is theoretically possible that they are determined at an earlier stage. A number of findings suggest that it is unlikely that pluripotent stem cells are derived only from early embryonic germ cell progenitors. Firstly, the day 6.5 *p.c.* primitive ectoderm is capable of self-renewal following tissue destruction, suggesting that allocation of cells to different lineages has not occurred by this stage (Snow and Tam, 1979). Teratocarcinomas have been formed from embryos homozygous at the *W* locus, which causes severe deficiency of germ cells in mice (Mintz, Cronmiller and Custer, 1978). And thirdly, up to four individual blastomeres from each 16- to 20-cell embryo have produced colonies with an ES-like cell morphology (Eistetter, 1989); these four blastomeres could not all be progenitors of the subsequent germ cell lineage.

1.4.2 Cultured Pluripotent Stem Cells: Normal or Epigenetically Modified Cells ?

There are two differing views proposing as to how it may be possible to isolate permanent cell lines of undifferentiated morphology from early embryos (discussed by Martin, 1980; Robertson and Bradley, 1986). Either, stem cells represent cells that have undergone a (reversible) epigenetic modification, or they are normal embryonic cells that continue to proliferate until they receive the appropriate developmental signals to differentiate. There are arguments for and against both of these hypotheses.

Cell transformation events have been responsible for the establishment of somatic cells into tissue-culture as permanent cell lines; whereby the cells undergo a "growth crisis" and initially lose proliferative potential, followed by the appearance of genetically altered (typically tetraploid) immortalised cells, of an apparently unchanged morphology (Todaro and Green, 1963; Loo *et al.*, 1987). Pluripotent stem cell lines have in common with transformed cell lines an apparently unlimited potential to proliferate *in vitro*. However, in the process of establishment, the ES cells do not undergo a "growth crisis" event, typical of transformed somatic cell lines (Suda *et al.*, 1987). Although EC cells do possess relatively more karyotypic abnormalities compared to ES cells, these generally only include various trisomies, chromosomal losses, partial deletions or translocations and the cells generally remain pseudodiploid. Chromosomal abnormalities may arise due to high selection pressures imposed upon cells. The EC cells within serially transplanted teratocarcinomas may be selected over long periods for a more malignant cell type, which may be aneuploid (Iles and Evans, 1977). *In vitro* EC cells grown without feeders no longer require exogenous growth factors for proliferation and lose their capacity for extensive differentiation (Heath, 1983). Isolation of ES cells without feeders may place immense pressure on the stem cells, particularly on cells derived from female embryos and account for the finding that such ES cells were transformed to a tetraploid state (Nichols *et al.*, 1990).

Because EC cells have gone through an intermediate tumour phase, they have been considered developmentally abnormal (Robertson, 1986). Indeed, many lines may be so (see section 1.2.8). Even apart from aneuploid cell lines, high contributions from EC cells have often been incompatible with normal embryonic development (Fujii and Martin, 1983) which is the critical test for normalcy. There are notable exceptions, however (*e.g.* METT-1), where EC cells have entered the germline of chimaeras (Mintz and Cronmiller, 1981; Stewart and Mintz, 1981). Such a reversion of formerly malignant EC cells might imply that any tumour "transformation" event to have been of a non-mutational, epigenetic nature (Mintz,

1978). That is, a (reversible) change in gene expression (rather than a change in chromosome structure) brought about from neoplastic conversion, triggered by an abnormal environment.

Another factor arguing against a mutational change, is the ease with which teratocarcinomas can be induced in inbred strains of mice by transferring embryos to an extra-uterine site (Mintz, 1978). However, unknown genetic factors in the graft-receiving host are important in the permissiveness of teratocarcinogenesis (see section 1.2.3). Although the establishment of EC cells from solid tumours has been difficult, because of the low numbers of stem cells amongst the vast assortment of differentiated cells (Evans, 1972; 1981), the culture of ascitic embryoid bodies has often resulted in the immediate isolation of EC cell lines (Kahan and Ephrussi, 1970). The rapid isolation of ES cells directly from embryos (in the absence of any obvious signs of cellular transformation: Suda *et al.*, 1987) from (perhaps) any mouse strain, has been suggested to indicate that these cells are true homologues of the early embryo cell phenotype (Robertson, 1986). Certainly, they participate in normal embryogenesis at a high frequency (Evans *et al.*, 1985).

If (embryonic) stem cells do not undergo "transformation" or any change in gene expression, how might such cells arise ? The second hypothesis (Martin, 1980; Robertson and Bradley, 1986) proposes that growth of the ICM/primitive ectoderm tissue may be normally suppressed by (embryonic or uterine) factors. This tissue is under fine regulatory control *in vivo*; with mechanisms compensating for changes in cell number arising from either embryo aggregation (Lewis and Rossant, 1982) or cellular damage (Snow and Tam, 1979). When the architecture of the embryo is disrupted, either in tissue-culture or ectopically, the undifferentiated cells may be able to "escape" normal developmental control and proliferate further. If this is true, then stem cells may be like normal embryonic cells that are naturally programmed to divide, until they receive appropriate signals to differentiate; for example, when re-introduced into an early embryo. This explanation ignores the fact that all ES cells and those EC cell lines with extensive differentiative capacities, are feeder-dependent; requiring a differentiation inhibitory factor to ensure proliferation of the pluripotent stem cells. And recent evidence suggests that extra-embryonic tissues of peri-implantation mouse embryos synthesise DIA/LIF which presumably interacts with the ICM and primitive ectoderm tissues in a paracrine manner, maintaining the undifferentiated phenotype (Conquet and Brulet, 1990; Rathjen *et al.*, 1990a).

1.4.3 Summary

The best assessment of the true relation of EC and ES cells to normal embryonic cells, is to test their developmental potential in chimaeras. Some embryonic stage differences have been detected in the limited chimaeric analyses of EC cells. Although ES cells derived from the ICMs of late blastocyst-stage embryos appear to resemble the day 5.5 *p.c.* primitive ectoderm biochemically, their potency is more similar to that of the cells of the early ICM. It appears that stem cells may be isolated from any undifferentiated, pluripotent tissue within the embryo (blastomeres, ICM or primitive ectoderm) or the foetus (primordial germ cells). The origin (early embryo or germ cell) is not important; the important point is that both of these sources contain undifferentiated, pluripotent cells.

It remains unresolved as to whether stem cells are able to be established into culture as a consequence of some epigenetic change in gene expression, or if they represent the normal proliferative potential of pluripotent embryonic cells. In general, it has been suggested that EC cells fall into the former category, while ES cells fall into the second. Experimental work presented in chapter five of this thesis may challenge this concept.

1.5 PLURIPOTENT STEM CELLS IN THE STUDY OF EARLY MURINE DEVELOPMENT

Cultured pluripotent embryonic cells have provided a unique system for studying fundamental aspects of mammalian developmental biology. This has been realised through both *in vitro* models of embryogenesis and *in vivo*, with analysis of gene function in the entire animal following genetic modifications performed in the laboratory.

1.5.1 *In Vitro* Models of Embryogenesis

The availability in tissue-culture of pluripotent embryonic cells, which under appropriate culture conditions can be induced to differentiate in a pattern resembling the normal embryo (see sections 1.2.6 and 1.3.4), has aided the study of early developmental events. The aggregation of stem cells in suspension culture has provided a model in which to study the processes involved in embryo compaction (Littlefield, 1989). The complex embryoid bodies formed from the differentiation of ES cells, may provide an *in vitro* model to analyse the events involved in the

development of the haematopoietic system (Risau *et al.*, 1988; Wiles and Keller, 1991).

Certain EC cell lines may differentiate to specific cell types with the appropriate chemical inducers and culture conditions; for instance, into parietal or visceral endoderm, cardiac muscle, neuronal tissue, etc. (see section 1.2.6). Although the cells may have been subjected to an embryologically inappropriate signal, controlled differentiation may provide tissue-culture models for differential gene expression. Qualitative differences between undifferentiated stem cells and differentiated cell populations in the expression profiles of proteins (Lovell-Badge and Evans, 1980), homeobox genes (Eistetter, 1987) and oncogenes (Lockett and Sleigh, 1987) may be potentially important in regulating these processes. cDNA clones have been isolated specific for undifferentiated stem cells (Stacey and Evans, 1984) and differentiated parietal endoderm cells (Kurkinen *et al.*, 1983).

X chromosome inactivation has been studied in tissue-culture. Some female EC cell lines normally possess two active X chromosomes, with one X chromosome becoming inactivated upon *in vitro* differentiation (McBurney and Strutt, 1980). However, when EC cells were exposed to retinoic acid and induced to form parietal endoderm, there was no preferentialinactivationof the paternal X chromosome as would be expected during normal embryo development (McBurney and Strutt, 1980). Cytogenetic studies with several parthenogenetically-derived female ES cell lines, each possessing a different-sized deletion of one X chromosome, have located a region on the X chromosome, which must be present on more than one X chromosome, in order for X inactivation to occur during the induction of *in vitro* differentiation (Rastan and Robertson, 1985).

An octomer binding transcription factor (known either as Oct-4 or Oct-3 by Scholer *et al.*, 1989 and Okamoto *et al.*, 1990; respectively) has been found to be present in undifferentiated stem cells and which disappears once the cells are induced to differentiate. The Oct-3 gene is developmentally regulated during murine embryogenesis, with Oct-3 expression in the pluripotent cells of the primitive ectoderm rapidly becoming down-regulated during gastrulation. The Oct-3 gene is also expressed in primordial germ cells and in the female germline (Rosner *et al.*, 1990).

Many antibodies which have been used to study the pre-implantation mouse embryo have been raised using EC cells as the immunogen, because of the similarities in origin and differentiation capacities between EC cells and early embryos. Some useful monoclonal antibodies have been produced which react with embryos in a stage- and/or tissue-specific manner (see Kimber, 1990 for a review).

Analysis of factors regulating the proliferation and differentiation of cultured stem cells (*e.g.* DIA/LIF) has increased the understanding of the regulatory

networks controlling normal murine development (Heath and Smith, 1988; Rathjen *et al.*, 1990a). Undifferentiated stem cells express very low levels of DIA/LIF, however, the activity of the genes is dramatically increased shortly after the induction of *in vitro* differentiation (Rathjen *et al.*, 1990a). A regulatory feedback mechanism has been proposed by the authors, with the differentiated cell progeny synthesising DIA/LIF to inhibit further differentiation of the stem cells. Similar paracrine interactions may occur during embryo development, between pluripotent cells and differentiated derivatives, as DIA/LIF transcripts have been detected in the extra-embryonic tissues of peri-implantation-stage mouse embryos (Conquet and Brulet, 1990; Rathjen *et al.*, 1990a).

1.5.2 *In Vivo* Analysis of Early Development

ES cells have been more applicable in the study of *in vivo* development than EC cells because they represent a more normal embryonic lineage. The capacity of ES cells to be maintained in long term culture, has allowed for the genetic manipulation and subsequent clonal selection of cells carrying the desired genetic modification, before using the stem cells to re-constitute an animal (Gossler *et al.*, 1986; Robertson *et al.*, 1986).

A recent approach increasing our understanding of the developmental function of specific genes, has been to introduce small mutations into genes by "gene targeting" whilst the ES cells are in culture, followed by analysis of the resultant phenotype in the chimaera or the F_1 (heterozygous) or F_2 (homozygous) ES cell-derived progeny. Introducing DNA into ES cells has also increased the potential of identifying novel developmentally regulated genes, by insertional mutagenesis.

The study of developmental mutations in existing mouse stocks has been aided by the isolation of ES cell lines from embryos homozygous at the mutant locus. For example, the differentiation of stem cells derived from embryos homozygous for the embryonic lethal t^{w5} haplotype (at the *t*-Complex) into teratocarcinomas composed of a wider range of tissues than occurs naturally by the time the embryos die *in vivo*, suggests than the mutation does not result in generalised cell death, nor does it interfere with differentiation *per se* (Magnuson *et al.*, 1982; 1983). In combination with the finding that homozygous t^{w5} embryos aggregated with +/+ embryos resulted in abnormal chimaeric foetuses, it has been suggested that the t^{w5} cells are unable to differentiate in an organised manner in response to the normal developmental signals from the host embryonic cells (Magnuson *et al.*, 1983).

1.5.2.1 Insertional Mutagenesis

The insertion of foreign DNA into the cellular genome may cause mutational changes by disrupting the function of an endogenous gene. In some cases, the DNA may insert into genes that play an important role in development and as a consequence, may perturb normal embryogenesis. Furthermore, the introduced DNA may serve as a "tag" for molecular cloning of the affected gene. Insertional mutations have been induced in mice by microinjection of cloned DNA into the pronucleus or by infection of embryos or ES cells with retroviruses (see reviews by Gridley, Soriano and Jaenisch, 1987; Jaenisch, 1988).

Retroviral vectors have been used to mutagenise cultured ES cells to facilitate the isolation of hypoxanthine guanine phosphoribosyl transferase-deficient variant cells (Kuehn *et al.*, 1987). A similar approach has lead to the detection of a recessive embryonic lethal mutation (413.d: Conlon, Barth and Robertson, 1991). It has been shown that the mutation does not operate in a cell autonomous manner, as ES cells derived from homozygous 413.d embryos have been "rescued" in all embryonic lineages in chimaeras and the homozygous stem cells have also entered the germline (Conlon *et al.*, 1991). It is hoped that cloning of the locus containing the inserted retroviral sequences, will lead to the identification of a gene expressed at the gastrulation stage of embryogenesis (Robertson, 1991).

A potentially more efficient approach in identifying (and mutating) novel developmentally regulated genes, may be the use of reporter gene constructs (termed "enhancer trap" and "gene trap" or "promoter trap") introduced into ES cells (reviewed by Rossant and Joyner, 1989). Enhancer trap vectors possess a bacterial ß-galactosidase (*lac Z*) gene linked with a minimal promoter. The *lac Z* gene may be transcribed if the construct integrates adjacent to an activating enhancer element within the host stem cell genome. Translation produces a *lac Z* protein which may be visualised by histochemical staining of chimaeric foetuses (Gossler *et al.*, 1989). Temporally and spatially restricted patterns of ß-galactosidase staining may indicate that the enhancer modulates the expression of an endogenous gene of developmental significance. In gene trap constructs the *lac Z* gene, lacking its own promoter, may be activated as a fusion transcript during development in the chimaeric foetus if the vector integrates within an endogenous gene following electroporation into ES cells. Restricted patterns of staining detecting the resultant *lac Z* fusion protein in the chimaeric foetus may indicate that the endogenous gene is developmentally regulated. Molecular cloning of the marked endogenous gene will be aided by the *lac Z* DNA sequences that are physically linked to the integration site. In addition, insertion of gene trap constructs are likely to generate a mutation in the host gene, which may be further analysed by breeding from the chimaera (Gossler *et al.*, 1989).

1.5.2.2 Gene Targeting

Gene targeting involves the site-directed recombination between introduced, cloned DNA sequences and homologous chromosomal sequences. Mammalian cells do possess the enzymatic machinery to generate this homologous recombination event, however, it occurs at a very low frequency; with the majority of the foreign DNA integrating at random sites within the chromosome (reviewed by Capecchi, 1989; Frohman and Martin, 1989). ES cells have proved amenable to methods of introducing DNA into cells (commonly by electroporation) and to the selection strategies devised to identify and propagate these rare targeted cells.

Initial efforts on homologous recombination in ES cells concentrated on the X chromosome-linked, hypoxanthine guanine phosphoribosyl transferase (*HPRT*) gene because of the hemizygous state in male cells and because of the directly selectable phenotype for, or against, the presence of the gene in tissue-culture. The *HPRT* gene has been both inactivated (Thomas and Capecchi, 1987) and corrected from a mutant ES cell line, with the genetic correction having been transmitted through the germline in chimaeras (Thompson *et al.*, 1989).

Few genes, however, can be altered to provide a directly selectable phenotype *in vitro*. Because experiments using the *HPRT* gene have shown that the frequency of random integration of DNA exceeds that of homologous recombination by a factor of a 1000-fold or more (Thompson *et al.*, 1989) efficient vector design and selection strategies have had to be devised to enrich for those cells containing these rare targeted events (for discussion of the various strategies used see reviews by Capecchi, 1989; Frohman and Martin, 1989; Robertson, 1991). Commonly, gene targeting vectors have contained the neomycin resistance (neo^R) gene introduced into a homologous sequence of DNA. The neo^R gene may have the dual function of providing a selectable marker *in vitro* (conferring resistance to the drug G418) in addition to disrupting the coding sequence of the targeted gene (Thomas and Capecchi, 1987).

Some of the refined strategies that have been utilised to enrich for homologous recombination events include the use of promotorless vectors, positive-negative selection and so-called "hit and run" strategies. Not all of the clones selected by these strategies will have been targeted and it has been necessary to verify homologous recombination with polymerase chain reaction and/or Southern blotting analyses.

The use of promoterless selection vectors (Jasin and Berg, 1988; Schwartzberg *et al.*, 1989; Riele *et al.*, 1990) is a strategy which is only applicable to target genes which are transcriptionally active in ES cells. The vector design is such,

that with homologous recombination the selection marker (*e.g.* the *neo*^{R} gene) is activated by the endogenous cellular promoter and may enrich for homologous recombination by a factor of 100-fold (reviewed by Robertson, 1991).

A very successful scheme has been the so-called "positive negative selection" devised by Mansour and associates (1988). This strategy has proved extremely useful for recovering mutations in genes that are not expressed in cultured ES cells (Johnson *et al.*, 1989; Thomas and Capecchi, 1990; McMahon and Bradley, 1990). With positive-negative selection, the replacement vector comprises a positive selection marker (the *neo*^{R} gene) and distal to the region of homology, a negative selection marker (the herpes simplex virus thymidine kinase - *HSV-tk* - gene). As random integration has been observed to occur via the ends of the introduced DNA (Capecchi, 1989), this results in the inclusion of the *HSV-tk* gene into the chromosome. Such cells are resistant to G418 (G418^R) (*i.e.* positive selection: indicating that the cells had integrated foreign DNA) but are susceptible to drugs such as ganciclovir (GANC) or deoxy-fluoro-arabinofuranosyl-iodouridine (FIAU), as the *HSV-tk* gene metabolises these precursors into toxic nucleotide analogs. And so, cells that have undergone a random integration event are negatively selected. With homologous recombination on the other hand, the *HSV-tk* sequences are cut out of the vector during the process of integration and thus, the targeted cells are G418^R and GANC^R/FIAU^R. A variation upon the positive-negative scheme has been reported, whereby negative selection was conducted without the addition of an exogenous drug. The *HSV-tk* gene in the vector was replaced instead with a diphtheria toxin A-fragment gene which, if transcribed after random integration, killed the cell and thus, enriched for homologous recombination (Yagi *et al.*, 1990). Typically, positive-negative selection has decreased the background of random integrants by 10- to 100-fold, with variation possibly reflecting differences in the capacity for homologous recombination at different genetic loci (reviewed by Robertson, 1991).

Insertion of the *neo*^{R} gene into the coding region of the target gene will inactivate the gene. Such null mutations have proved valuable in studying development (see below), however, for the detailed *in vivo* analyses of protein structure and function, more subtle (insertion, deletion, and substitution) mutations are needed. Recently, a two-step recombination procedure, termed "hit and run", has been utilised to introduce small mutations into the *HPRT* and *Hox-2.6* genes, without any selectable markers remaining in the successfully targeted event (Hasty *et al.*, 1991). Thus, no *neo* cassette will be present to potentially interfere with transcription of neighbouring genes and confuse interpretation of the altered allele's phenotype. An alternative approach to introduce small mutations without a selectable marker, has been to microinject DNA directly into ES cells followed by screening the cells using the

polymerase chain reaction. Although technically demanding, homologous recombination has been reported to occur at a relatively high frequency (1/150 cells microinjected: Zimmer and Gruss, 1989).

Following the selection of genetically modified cells *in vitro*, phenotypic analysis of the altered allele may be achieved *in vivo* by the examination of the chimaera itself or through the germline, in the ES-derived F_1 (heterozygous) or intercrossed F_2 (homozygous) progeny. Alternatively, some applications may be better addressed by altering both alleles *in vitro* and studying the pattern of stem cell differentiation in culture (Riele *et al.*, 1990).

Gene targeting experiments have provided interesting data on the developmental function of specific genes (see review by Robertson, 1991). For example, inactivation of the *IGF-II* gene resulted in normally proportioned, but miniature mice in the heterozygous F_1 generation (DeChiara, Efstratiadis and Robertson, 1990). Subsequent breeding studies have found the *IGF-II* gene to be parentally imprinted (DeChiara, Robertson and Efstratiadis, 1991). Partial functional redundancy of certain genes may explain the results of Joyner and colleagues (1991) where homozygous *en-2* null mutant mice exhibited defects in only a subset of the tissues known to express the gene. Similar observations have been made for the *Hox-1.5* gene (Chisaka and Capecchi, 1991). Although the genetics are different, the pathology of homozygous-deficient *Hox-1.5* mice is very similar to the human congenital disorder DiGeorge's syndrome and thus, these mice might provide a useful disease model (Chisaka and Capecchi, 1991).

1.5.3 Summary

This section has reviewed some of the applications of stem cells in the study of development. Both EC and ES cells have been utilised in studies on *in vitro* differentiation; the restricted capacity of some EC cell lines to differentiate only into one other cell type upon exposure to certain drugs, has been beneficial in generating homogeneous cell populations for biochemical and molecular analysis. Only ES cells, however, have allowed for the *in vitro* manipulation of the mouse germline. Random insertional mutagenesis has identified new developmentally significant genes. Future use of reporter constructs introduced into ES cells, followed by screening of "interesting" staining patterns in the chimaeric foetus, undoubtedly will uncover many more novel genes. The precise disruption of several known endogenous genes by homologous recombination (or gene targeting) has already revealed the function of these genes within the mutant animal. This novel genetic approach to studying development is becoming more sophisticated, with subtle modifications being

introduced into genes to alter protein structure, rather than just creating null mutations. Furthermore, it appears to be feasible to target any known gene within the genome. There is also the possibility of generating mouse models for human genetic disorders. Apart from generating mutant mice for the study of development or disease, there is the potential of using gene targeting in ES cells to introduce "transgenes" into the genome to produce novel proteins, in the milk, for example. The major advantage of utilising ES cells compared to conventional methods of introducing foreign DNA sequences into the germline (see Jaenisch, 1988), rests in the ability to select the stem cell clone carrying precisely the desired modification *in vitro*. Methods such as pronuclear injection, allow no control over the chromosomal site of DNA integration nor the number of copies introduced. Thus, expression of the transgene may be highly variable in these animals. Following manipulation, the ES cells retain the capacity to colonise the germline in chimaeras and with subsequent backcrossing by conventional breeding, F_1 progeny, heterozygous for the modified gene, are produced. Even allowing for the extra generation, genetic manipulation of cultured ES cells is a more efficient means of producing mutant and transgenic animals than pronuclear injection.

1.6 EMBRYONIC STEM CELLS FROM OTHER MAMMALIAN SPECIES

The mouse has provided a model system for the application of the ES cell technology; demonstrating a powerful alternative for transgenesis and genetic manipulation of the germline to that of zygote pronuclear injection of foreign DNA (see section 1.5.2.2). The prospect of further extending this technology into modifying genetic traits of economic importance in commercial domestic species, has principally lead to the intense interest in isolating ES cells from animals such as pigs, sheep and cattle. In these species, there is the additional exciting possibility, as suggested by Smith and Wilmut (1989), of performing nuclear transfer with donor stem cell nuclei and producing an animal that is entirely ES cell-derived. This by-passes the chimaeric generation and the subsequent time-consuming backcrossing necessary to produce a homozygous individual. Such a technology would be of tremendous importance in future livestock breeding.

The prime interest in obtaining ES cells from other laboratory species, is more concerned with providing new animal disease models and alternative systems for the study of developmental gene function, which for some applications may be better addressed in species other than the mouse.

This section reviews published attempts to isolate ES cells from other rodent and farm animal species.

1.6.1 Rodents

The only other small laboratory species from which embryo-derived pluripotent cells have been isolated is the Syrian golden hamster (*Mesocricetus auratus*; Doetschman, Williams and Maeda, 1988). Stable cell lines of an undifferentiated morphology were obtained from 1% (4/409) of intact blastocysts grown on mouse embryonic fibroblasts. These cells possessed a normal chromosome number and exhibited extensive differentiation in suspension culture, forming cystic embryoid bodies (Doetschman *et al.*, 1988). On the basis of this *in vitro* differentiative capacity, these cells have been deemed pluripotent by the authors and hence, termed "hamster ES cells"; however, no published data is available on the capacity of these cells to participate in normal embryo development *in vivo*.

1.6.2 Farm Animals

The difficulties experienced in the attempts to isolate stem cells from farm species may be due to fundamental species differences in embryology, compared to the mouse and/or to species specific culture requirements.

Whilst the embryology of the farm species follows a similar pattern, the events after blastulation and leading up to implantation are quite different when compared to the mouse. With particular regard to ES cell isolation, the ICM/primitive ectoderm of the mouse blastocyst is a more proliferative tissue (Gardner and Beddington, 1988) than the equivalent "embryonic disc" in farm animals (McWhir *et al.*, 1991). Thus, the expectation that these undifferentiated cells would not be easily cultured *in vitro* has been evident for the sheep (Handyside *et al.*, 1987), pig (McWhir, 1988) and cow (Schellander *et al.*, 1989). ES-like cells (so-called since the pluripotency of this cell lineage has not been proven) from these species display much slower rates of growth *in vitro* compared to the mouse and this is probably a reflection of the quiescent nature of the embryonic disc *in vivo*. As a consequence, passage intervals in culture are typically extended to between five (Evans *et al.*, 1990) and 10 days (Piedrahita, Anderson and BonDurant, 1990a).

There has been an effort to identify embryonic stages in farm animals comparable to the day 3.5 *p.c.* mouse embryo; particularly to identify blastocyst-stages before endodermal delamination from the blastocoelic surface of the ICM has occurred. This may be important, as Robertson (1987) has observed in the mouse

that the presence of endodermal cells *in vitro* promotes further differentiation within the culture. In the pig, endoderm differentiation does not take place until day 10-12 *post oestrus* (*p.o.*) (Notarianni *et al.*, 1991). However, endoderm differentiation appears to commence at relatively earlier blastocyst-stages in the sheep (day seven: Handyside *et al.*, 1987; McWhir *et al.*, 1991) and cow (day eight: Betteridge and Flechon, 1988). Thus, there have been difficulties in obtaining a large population of ICM cells that have not been influenced by endoderm, especially in the sheep. Workers that have been successful in isolating ES-like cells (from the pig) have tended to isolate the ICM either immunosurgically (Piedrahita *et al.*, 1990a) or mechanically (Notarianni *et al.*, 1990b) in order to remove any influences of differentiated cells from the cultures.

Isolation into tissue-culture of ES cells from these species has been extremely difficult because of problems associated with their inherent slow growth *in vitro*, further complicated by the fact that these cells are morphologically unstable (Handyside *et al.*, 1987; Piedrahita *et al.*, 1990b; McWhir *et al.*, 1991) with very few ES-like colonies surviving beyond the third passage. Typically, putative ES cell colonies have been observed to progressively differentiate into large, flat epithelial-like cells, or to simply die from the culture (McWhir *et al.*, 1991). McWhir (1988) was only able to maintain his porcine ES-like cell line in a stable state following transformation with the polyoma virus middle T antigen. However, recent reports (see below) have described the isolation of stable cell lines of primary ectodermal origin from farm species, which may be the sought after ES cells.

There might be species specific culture requirements for farm animal stem cells, but there are no reports of major innovations along these lines - methods have been similar to those utilised for the mouse. Medium supplemented with anti-differentiation factors, such as mouse LIF (McWhir *et al.*, 1991) or 60% BRL-conditioned medium (Handyside *et al.*, 1987) were not able to prevent the differentiation of ovine ES-like cells in these studies. A comprehensive study concerning the influence of the feeder cell layer has shown that mouse STO fibroblasts remained superior for the isolation of porcine ES-like cells (Piedrahita *et al.*, 1990a). Ware and First (1988) reported a combination of mitotically inactivated BRL cells and mouse primary foetal fibroblasts enhanced the proliferation of embryonic cells derived from pigs, sheep and cattle, however, this effect has not been repeated (Piedrahita *et al.*, 1990a).

1.6.2.1 Pig (*Sus scrofa*)

Two independent groups have isolated stable ES-like cell lines from day seven to day nine *p.o.* pig blastocysts (Evans *et al.*, 1990; Notarianni *et al.*, 1990a; 1990b; 1991; and Piedrahita *et al.*, 1990a; 1990b). The cells from both groups appear similar, but somewhat different compared to mouse ES cells. They grow instead as flattened colonies on the STO feeder cells. Individual ES-like cells do, however, possess large translucent nuclei with prominent nucleoli. Interestingly, differences in cell size between independently isolated ES-like cell lines has been reported (Notarianni *et al.*, 1990b). This may indicate that these cell lines are not all of the same embryonic lineage. *In vitro* differentiation into cellular derivatives representative of the three primary germ layers has been observed (Evans *et al.*, 1990). However, Piedrahita and colleagues (1990b) have not been able to induce their porcine ES-like cell lines to differentiate *in vitro* or *in vivo*; with the cells not having formed embryoid bodies, teratomas or chimaeras (0/21 piglets born). It has not been reported whether the porcine ES-like cell lines isolated in the Evans' laboratory are capable of participating in normal embryo development.

1.6.2.2 Sheep (*Ovis aries*)

The isolation of one stable ES-like cell line derived from a day eight *p.o.* intact blastocyst has been reported (Notarianni *et al.*, 1990a; 1991). These cells display similar morphology and growth characteristics to the porcine ES-like cell lines and spontaneously differentiate on reaching confluence into a range of cell types (Notarianni *et al.*, 1990a). However, it has not been reported if these ovine ES-like cells are pluripotent and capable of forming chimaeras. Although ICMs isolated from day seven to day nine *p.o.* blastocysts have been reported to give rise to more outgrowths and primary ES-like colonies compared to intact blastocysts, no colonies maintained a stable ES-like morphology for more than two or three passages (Notarianni *et al.*, 1990a; 1991). Only epithelial-like cell lines have been established from ovine ICMs (Piedrahita *et al.*, 1990b).

1.6.2.3 Cattle (*Bos tauris*)

Few reports have been published for the cow. Bovine cell lines with a stem cell-like morphology have been isolated, however, no information is available on their culture history, or on their *in vitro* and *in vivo* differentiative

capacities (Evans *et al.*, 1990). Similar cells have apparently been cultured to the fourth passage (Strelchenko, Saito and Niemann, 1991).

1.6.3 Summary

It has not been a trivial task to isolate ES cells from other species. Although ES-like cells have been obtained in the hamster, pig, sheep and cow, none have yet been shown to be pluripotent *in vivo*. Many difficulties encountered in the isolation of stem cells from farm animals may be due to inherent species differences in early embryology and in the behaviour of pluripotent cells *in vivo*. Optimal culture conditions have yet to be defined for these species.

CHAPTER 2

GENERAL MATERIALS AND METHODS UTILISED FOR MURINE EMBRYONIC STEM CELLS

This chapter outlines the general materials and methods common to the subsequent experimental chapters on murine ES cells. Sections detail the tissue-culture facility and standard procedures, the production of embryos from mice and the techniques used for ES cell isolation and routine culture. The basic protocols employed in this thesis, were to a large extent based upon the methodologies described by Robertson (1987). A section describes the micro-manipulative technique used for the production of chimaeric mice, via blastocyst injection. A final section describes the statistical analyses performed on the data presented in this thesis. Each experimental chapter contains a more specific account of the procedures and treatments that were utilised, especially in chapters six and seven concerning the immunohistology of ovine embryos and the attempts to isolate stem cells in the sheep.

2.1 GENERAL TISSUE-CULTURE

This section describes the equipment within the tissue-culture facility, the media used in the isolation and maintenance of murine ES cells and the procedures for the preparation of the STO feeder cell layers, which were used for the co-culture of embryos and ES cells.

2.1.1 The Tissue-Culture Facility

All experimental studies on stem cell isolation reported in this thesis were conducted at IAPGR, Roslin, in a laboratory established solely for sterile ES cell culture. The basic equipment in this laboratory included: a vertical flow tissue-culture cabinet (Gelaire); two humidified, 5% CO_2 (in air) incubators (Flow Laboratories) set at 37°C; a bench-top centrifuge; a waterbath; an inverted, phase contrast microscope (magnification: x40, x100, x200; Nikon Diaphot) with a Nikon F-301 automatic camera (photographs taken on Pan F, 25 ASA film; Kodak); a binocular, dissecting microscope with transmitted illumination (magnification: x12-x100; M8, Wild) within

a perspex still-air cabinet; and one propane gas Bunsen burner, with a pilot flame, within each culture cabinet.

Medium was dispensed with plastic, disposable pipettes (Sterilin; Sterilin Ltd., or Costar; Northumbria Biologicals Ltd.; pipette sizes: 1ml, 5ml, 10ml and 25ml) utilising a rechargeable battery operated, hand-held pipette-aid (Drummond). A water-generated vacuum, with two waste traps, was utilised to aspirate spent medium through a tube connected to disposable, heat-sterilised, long-form glass Pasteur pipettes.

Glassware designated specifically for stem cell culture was washed without any detergents. Glassware was soaked for one to two days in a bucket of double-distilled water and rinsed several times in ultra-purified "milli-Q-water" (Millipore filtration system). After draining, general glassware was heat-sterilised (at 180°C for two hours) while bottles with screw on tops were autoclaved (at 101kPal for 20 minutes).

Standard sterile tissue-culture procedures were employed throughout. Personnel entry was restricted and no mice were handled in the tissue-culture laboratory. The majority of work was performed within the vertical flow cabinet. However, in the course of ES cell isolation, it was necessary to operate on the dissecting microscope within the still-air cabinet. With care, microbial contamination was kept to a minimum. Testing for mycoplasma-infected cells was performed by the AFRC Moredun Research Institute, Edinburgh and none was detected in these studies.

2.1.2 Tissue-Culture Media and Solutions

All tissue-culture medium was based upon a 1X strength of Dulbecco's Modification of Eagle's Medium (DMEM; Life Technologies) - a formulation without sodium pyruvate, but high (4500mg/ml) in glucose. The two media formulations used for ES cell culture are given in Table 2.1. The main difference was in the serum concentration. For ES cell isolation, medium contained 20% (v/v) serum (ES_{20}), while established ES cell lines were maintained in medium with only 10% (v/v) serum (ES_{10}) and supplemented with recombinant DIA/LIF (see section 2.4.1 and chapter 1.3.3.5). In both instances, serum comprised a 1:1 mixture of selected batches of foetal calf serum (FCS: Globepharm) and newborn calf serum (NCS: Sera-Lab). The same batch of each serum type was used throughout these studies. The most suitable serum, from several test samples provided by various suppliers, had been selected after testing their plating efficiency with an established ES cell line. This serum testing was conducted by Mary Jones whilst at the Department of Pathology, University of Edinburgh. Serum for mouse ES cell culture was not heat-inactivated.

Table 2.1: Media for murine embryonic stem cell culture

A.) Medium for murine embryonic stem cell isolation (ES_{20} medium)

64 ml	**Dulbecco's Modification of Eagle's Medium (DMEM)**
10 ml	**Foetal calf serum (FCS)**
10 ml	**Newborn calf serum (NCS)**
1 ml	**200mM L-glutamine**
1 ml	**100X non-essential amino acids (NEAA) stock**
1 ml	**ß-mercaptoethanol stock (final concentration:0.1 mM)**
13 ml	**Analar water**

That is, 20% sera in 100 ml of medium (Osmolarity: *c.a.* 290 mOs)

B.) Medium for murine embryonic stem cell maintenance (ES_{10} medium)

74 ml	**Dulbecco's Modification of Eagle's Medium (DMEM)**
5 ml	**Foetal calf serum (FCS)**
5 ml	**Newborn calf serum (NCS)**
1 ml	**200 mM L-glutamine**
1 ml	**100X non-essential amino acids (NEAA) stock**
1 ml	**ß-mercaptoethanol stock (final concentration: 0.1 mM)**
13 ml	**Analar water**

That is, 10% sera in 100 ml of medium (Osmolariy: *c.a.* 290 mOs)

To ES_{10} medium, 0.1% (v/v) human recombinant DIA/LIF supernatant was added (see section 2.4.1)

C.) 100X ß-mercaptoethanol stock

7 µl of ß-mercaptoethanol was dissolved in 10 ml of PBS (Table 2.2) and made fresh before addition to ES cell medium.

All media was filter sterilised (0.22µm) and stored at 4°C, warming to 37°C before use.

Table 2.2: Recipes for tissue-culture solutions

A.) Trypsin / EGTA solution (TEG)

To 1 *l* of Analar water, add:

7.0	g	**NaCl**
0.3	g	**$Na_2HPO_4.12H_2O$**
0.24	g	**KH_2PO_4**
0.37	g	**KCl**
1.0	g	**D-glucose**
3.0	g	**Tris [2-amino-2-(hydroxymethyl) propane-1,3-diol]**
1.0	ml	**Phenol red**

Dissolve ingredients and discard 100 ml of the salts solution, then add:

100 ml	**2.5% (10X) trypsin in modified Hank's balanced salts solution (Flow Laboratories)**
0.4 g	**EGTA (ethyleneglycol-tetraacetic acid)**
0.1 g	**PVA (polyvinyl alcohol)**

TEG was adjusted to pH 7.6, filter sterilised (0.22μm), aliquoted into 20 ml sterile, plastic universals (Sterilin) and stored at -20°C.

B.) Phosphate buffered saline (PBS)

Ca^{2+} and Mg^{2+} free PBS was prepared by dissolving pre-formulated tablets (Flow Laboratories) in Analar water. PBS was aliquoted into 500 ml bottles, sterilised by autoclaving and stored at room temperature.

In addition to serum, ES media were supplemented with 1% (v/v) of 200mM L-glutamine (Flow Laboratories) since this amino acid is unstable, 1% (v/v) of a 100X stock solution of non-essential amino acids (Flow Laboratories; the final concentrations of the NEAA added were: glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid 0.1mM each; L-proline, L-serine 0.2mM each) and 0.1mM ß-mercaptoethanol (Sigma)(Table 2.1).

It was found that the osmolarity of DMEM, as supplied by Life Technologies, was approximately 345mOs and was still considered very high after all of the above supplements and serum had been added (ES_{20}: *c.a.* 335mOs and ES_{10}: *c.a.* 340mOs). Although an initial study found no significant differences in the efficiency of ES cell isolation with media of varying osmolarities (D.N. Wells, results not presented), the plating efficiency of an established ES cell line was observed to have been optimal with medium of 290mOs (Dr J. McWhir, *pers. comm.*). Hence, in all of these studies, the osmolarity of both ES_{10} and ES_{20} media was reduced to around 290mOs by the addition of 13% (v/v) Analar water (Table 2.1).

Medium was filter-sterilised after preparation by utilising a 60ml syringe to push the medium through a glass-fibre pre-filter overlying a 0.22µm nitrocellulose filter, housed within a 47mm filter-holder (Millipore). Media were stored at 4°C and warmed to 37°C immediately before use. Medium older than two weeks was supplemented with 1% (v/v) of 200mM L-glutamine.

For both ES cell isolation and routine culture, **no** antibiotics were used. If a culture became infected (especially if by fungus or yeast) it was generally discarded from the laboratory (see section 2.6 for the analysis of the data in cases where individual cultures became infected during the course of ES cell isolation experiments). If, however, a particularly valuable culture was infected with bacteria, it was extensively washed in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS; Table 2.2) before medium containing 50 IU/ml penicillin (Na salt) and 50µg/ml streptomycin (Sigma) was added.

A 0.25% (v/v) trypsin, 0.04% (w/v) EGTA (ethyleneglycol-tetraacetic acid) solution was utilised in the sub-culture of both fibroblast and ES cell lines and for the disaggregation of ICM outgrowths and putative stem cell colonies, in the process of ES cell isolation. The powdered ingredients (Table 2.2) were of tissue-culture purity (purchased from Sigma or BDH) and kept solely for ES cell culture.

2.1.3 Preparation of STO Feeder Cell Layers

The STO fibroblast line (Martin and Evans, 1975) was routinely cultured in DMEM + 1% (v/v) 200mM L-glutamine + 5% (v/v) of both FCS and NCS

($DMEM_{10}$ medium) in tissue-culture flasks, commonly with a $25cm^2$ growth area surface (Costar; Northumbria Biologicals Ltd.). Once confluent, the fibroblasts were passaged, or sub-cultured, into fresh flasks. The old medium was aspirated from the flask and the cells washed once with 10ml of PBS. After the PBS was aspirated, 2ml of TEG was added and the flask incubated at 37°C for three minutes. After swirling the flask, so as to obtain a single-cell suspension (checked in the inverted microscope) the trypsin solution was neutralised by adding 3ml $DMEM_{10}$. Typically, a 1/10th aliquot (*i.e.* 0.5ml) of this suspension was added to 10ml of fresh $DMEM_{10}$ in a new flask. Under such a sub-culture regime, flasks reached confluency after four to five days. The sub-culture ratio and the number and size of the flasks seeded, was varied to suit the demand for STOs. The flasks were transferred into the CO_2 incubator with either their caps loosened or, tightened after being gassed whilst in the flow cabinet via a sterile, plugged Pasteur pipette connected to a cylinder containing 5% CO_2, 20% O_2 and 75% N_2.

Mitotically inactivated STO feeder cell layers were prepared using the following protocol. The old medium was aspirated from a sub-confluent flask of STOs and for a $25cm^2$ flask, replaced with 10ml of $DMEM_{10}$ containing 10µg/ml of mitomycin C (Sigma). Appropriate precautions were taken when handling this potential carcinogen. The stock solution was prepared by dissolving a 2mg vial of mitomycin C in 5ml of PBS (*i.e.* 400µg/ml) which was stored at 4°C for no longer than one month. The STO flasks were then incubated for three hours.

After treatment, the mitomycin C medium was aspirated and the STO cells were washed three times with 10ml of PBS, before being trypsinised with 2ml of TEG. After three minutes incubation, the STOs were dissociated into single-cells and the TEG was neutralised with 8ml of $DMEM_{10}$. The total 10ml suspension was transferred into a 20ml conical, plastic universal (Sterilin). A minute volume of the suspension was drawn into a Pasteur pipette and transferred to a haematocytometer, in order to count the number of cells. The STO cell suspension was centrifuged at 1000 r.p.m. for five minutes and the supernatant aspirated. The cell pellet was then disrupted by carefully flicking the tube and the cells were resuspended in $DMEM_{10}$ medium, to one of three alternative densities (see below).

Feeder cell layers were prepared in three sizes with differing surface-areas: either in microdrops under toxicity-tested, lightweight paraffin oil (BDH), in $1.75cm^2$ four-well plates (Nunc; Life Technologies) or in $25cm^2$ flasks (Costar; Northumbria Biologicals Ltd.). To enhance STO cell attachment, all culture surfaces were pre-treated with gelatin (porcine skin; Sigma). The gelatin was made as a 0.1% (w/v) solution in Analar water and sterilised by autoclaving. Microdrops were prepared by placing two rows of five 10µl drops of gelatin on 6cm diameter tissue-

culture dishes (Costar; Northumbria Biologicals Ltd.), overlayered with 5ml of paraffin oil. For wells and flasks, just enough gelatin was added so as to cover the culture surface. The gelatin was stored for about one hour at room temperature, before aspiration and replacement with the freshly prepared, inactivated STO cell suspension.

The STOs were plated at a density to ensure a confluent, uniform monolayer of cells. For microdrops, 20µl of a suspension containing 3 x 10^5 STO cells/ml was introduced; each (1.75cm^2) well, of a four-well plate, received 1ml of a suspension containing 1.5 x 10^5 cells/ml; while 25cm^2 flasks were seeded with 1 x 10^6 STO cells in 5ml of $DMEM_{10}$ medium. Feeders were generally used within five days, after which time, fresh feeder layers were prepared. The old medium was aspirated and fresh ES medium introduced, before the feeder layers were used for co-culture with embryos or ES cells.

2.2 PRODUCTION OF BLASTOCYST-STAGE MOUSE EMBRYOS

For ES cell isolation, blastocyst-stage embryos were recovered 3.5 days *p.c.* from two mouse genotypes - crossbred F_1 (C57BL/6 X CBA/Ca) females mated to F_1 (C57BL/6 X CBA/Ca) males and inbred 129/Sv-*CP* strain mice. The C57BL/6 and CBA/Ca mice were from existing inbred strains at IAPGR, Roslin, while the 129/Sv-*CP* strain was obtained from R. Quinney of the MRC Radiobiology Laboratory, Harwell, U.K.. This 129 sub-strain was derived from 129/Sv-Sl^J, *C*, *P*, however, over a number of generations the mutant Steel (Sl^J) locus had been bred out of the 129 genetic background. For blastocyst injection experiments, mice from the outbred MF1 strain were used (see section 2.5).

F_1 and MF1 mouse stocks were housed in a separate facility (SAU) to that used for the 129/Sv-*CP* strain (Hut 5a). The SAU had a light cycle of 14 hours light (04:00hrs to 18:00hrs) and 10 hours dark, whereas Hut 5a employed a 12 hour light (06:00 to 18:00) and 12 hour dark cycle. Mice were fed and watered *ad libitum*.

2.2.1 Superovulation

The hormones used to superovulate mice were pregnant mare's serum gonadotrophin (PMS, Folligon; Intervet) to increase follicle production and human chorionic gonadotrophin (hCG, Chorulon, Intervet) to induce ovulation artificially. Both PMS and hCG were prepared by dissolving the lyophilised powders in sterile 0.9% (v/v) NaCl, to give a final concentration of 50 IU/ml. The hormones were then aliquoted, stored at -20°C and replaced after two months.

Six to eight week old virgin females, from all three mouse genotypes (129/Sv-*CP* , F_1 and MF1), were injected intraperitoneally with five IU (*i.e.* 0.1ml) of PMS at approximately 13:00hrs. Forty-seven hours later, the same females were injected intraperitoneally with five IU (*i.e.* 0.1ml) of hCG. Females were then paired with stud males (of the appropriate strain) and checked for copulation plugs the following morning (designated day 0.5 *p.c.*). It was assumed that ovulation had occurred 12 hours after the administration of hCG, that is, around the mid-point of the dark cycle.

2.2.2 Uterine Recovery of Blastocysts

Females were killed by cervical dislocation on day 3.5 *p.c.*. They were laid out on their backs on absorbant tissues and the abdominal surface was soaked with 70% ethanol. The skin was pinched up with forceps and a small cut made across the midline. Grasping both sides of the cut firmly with forceps, the skin was pulled in opposing directions to expose the abdominal body wall, which was in turn cut open. The alimentary tract was displaced to one side to reveal the reproductive organs near the dorsal surface of the female.

The uterus, containing the blastocysts, was removed by cutting across the base of the cervix, but above the bladder, and cutting away the mesometrium membrane holding the uterus to the body wall. Both uterine horns were cut just below the junction with the oviduct and the uterus was placed on clean tissue. The uterine horns were cut from the cervix and any remaining mesometrium or adipose tissue was trimmed away. A 25-gauge needle, attached to a syringe containing $DMEM_{10}$ + antibiotics (see section 2.1.3), was inserted 2-3mm inside the tubal end of the uterine horn and was grasped firmly with forceps. Approximately, 1ml of medium was flushed through each horn and collected in a 6cm dish (Costar; Northumbria Biologicals Ltd.).

The dish containing the uterine flushings was transferred to the dissecting microscope and under low magnification, was searched for embryos. Blastocysts were aspirated into a mouth-controlled, hand-pulled, plugged Pasteur pipette. This was constructed by rotating the pipette over a Bunsen burner, to firstly soften the glass. After withdrawal from the flame, the pipette was quickly pulled to produce a fine, square tipped capillary, approximately 6cm long and with an internal diameter of 150-200μm. A modified Pasteur pipette bulb was utilised as an adaptor to connect the end of the pulled pipette to a length of rubber tubing with a fitted mouthpiece. Fine control over the movement of embryos within the pipette was aided by firstly aspirating several minute volumes of medium interspaced with air bubbles.

The embryos were then collected from the flushings and pooled into a 25µl drop of ES_{20} medium under oil. This helped their subsequent location, before random allocation to the various experimental treatments.

2.3 ISOLATION OF MURINE EMBRYONIC STEM CELLS

This section describes the procedures that were utilised to isolate ES cells from intact, blastocyst-stage mouse embryos; that is, both day 3.5 *p.c.* and delayed blastocysts (see chapter 3.2) as, apart from the timing of the disaggregation of the ICM outgrowth, the procedures used were identical. The approach of isolating murine ES cells from day 5.5 *p.c.* primitive ectoderm is provided in chapter 4.2. ES cells are typified as small, rounded cells with a large nuclear to cytoplasmic ratio, containing one or more prominent nucleoli and no overtly specialised cellular structures. That is, characteristics of a typically undifferentiated cell morphology.

ES_{20} medium (Table 2.1) was used for ES cell isolation. After embryo recovery, the blastocysts were co-cultured on STO feeder layers prepared in microdrops (section 2.1.3). The medium was changed on these microdrops two to three hours before the embryos were introduced. Using a sterile, hand-pulled Pasteur pipette, one blastocyst was transferred to each, coded microdrop. As the main statistic from these studies was the efficiency of ES cell isolation from the various treatments (see section 2.6), microdrops were an essential part of the experimental design; whereby the progress of each embryo was monitored individually. An additional advantage of using microdrops was that any ES cell colonies arising from a single embryo were pooled together, without the necessity for cloning as required for group-cultures.

Blastocysts explanted into the microdrops, hatched from the zona pellucida and attached to the STOs after 24-36 hours by the outgrowth of the trophectodermal cells (figure 2.1a). These cells spread out and differentiated, to form a monolayer of large, flat trophoblast cells. As a consequence of this disorganisation, the ICM became exposed to the culture environment and appeared initially as a small nest of cells in the centre of the trophoblast outgrowth after two to three days (figure 2.1b). After embryo attachment, the medium on the microdrops was replaced daily. The ICM cells continued to proliferate and the outgrowths were allowed to grow to a suitably large size (determined by experience; see figure 2.1c), but without signs of endoderm differentiation, before they were disaggregated. Such outgrowths were obtained after four to five days of culture with day 3.5 *p.c.* blastocysts. Delayed blastocysts, however, generally required a six day culture interval before they reached a suitable size, due to their slower rates of *in vitro* growth initially. Variability between

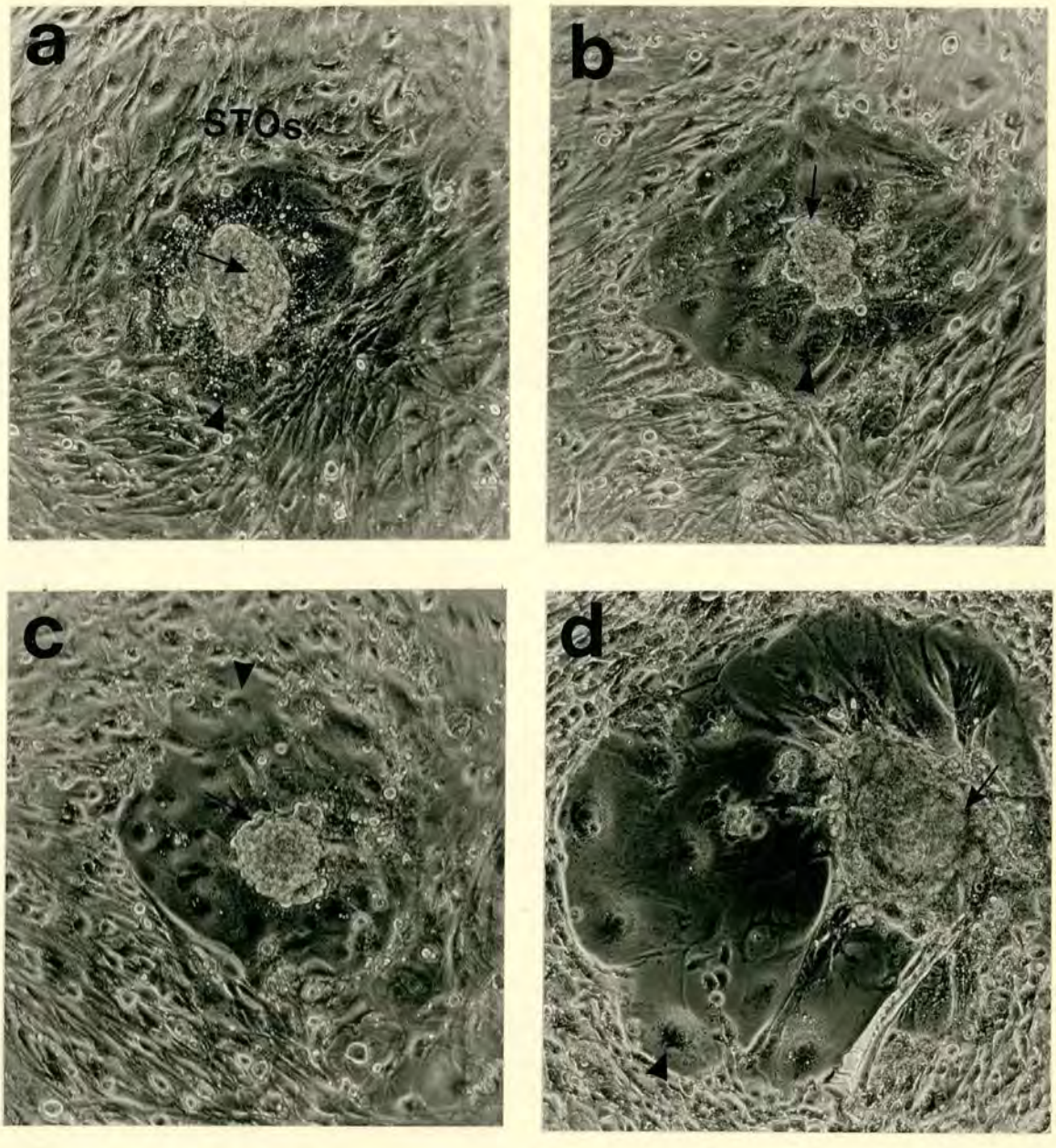


Figure 2.1: Attachment and outgrowth of day 3.5 *p.c.* blastocyst-stage mouse embryos on feeder cell layers. In the above panels, the ICM is indicated by arrows and the trophoblast by arrowheads. Embryos hatch from the zona pellucida and attach to the STO feeder cells via the trophectoderm after 24-36 hours of culture (a). In the process of attachment the blastocysts collapse, sequestering the ICM (a). Subsequently, the trophectoderm differentiates into giant trophoblast cells (a) which grow out radially to eventually expose the ICM after two or three days (b). The ICM outgrowth proliferates as a three-dimensional mass of cells and is disaggregated once it has reached a size similar to that in (c) after a total of four to five days *in vitro*. ICM outgrowths were disaggregated before they had begun to differentiate into endoderm (d). Photographs were taken using phase contrast microscopy. (Magnification: x100)

embryos existed in the rate of ICM growth and thus, each embryo was monitored and assessed daily, with the inverted microscope. If cultured without intervention, cells of the embryonic outgrowth differentiated (figure 2.1d).

2.3.1 Disaggregation of ICM Outgrowths

Two to three hours before ICM outgrowths were disaggregated, the cultures were re-fed with fresh medium. The disaggregation procedure was performed by transferring the culture dish to the dissecting microscope and with a blunt glass probe, prepared from a flame-polished, hand-pulled Pasteur pipette, the ICM was "picked off" from the surrounding layer of trophoblast. With a mouth-controlled, drawn-out Pasteur pipette, the ICM was transferred to a 20µl wash-drop of TEG on a 6cm dish (Costar)(without oil) before being placed into a fresh drop of TEG and incubated at 37°C for five minutes. Returning the dish to the dissecting microscope, a finely pulled Pasteur pipette, with a tip diameter one-fifth the size of the ICM clump, was used to mechanically break-up the partially digested outgrowth into several small pieces, each comprising around 15 cells. All of the cellular pieces from one embryo were then transferred to a fresh STO microdrop containing ES_{20} medium. The microdrop was labelled with the coded embryo number and this disaggregation was termed "passage one". Approximately six hours after disaggregation, the medium was changed on the culture to remove any dead cells and any residual traces of the trypsin solution.

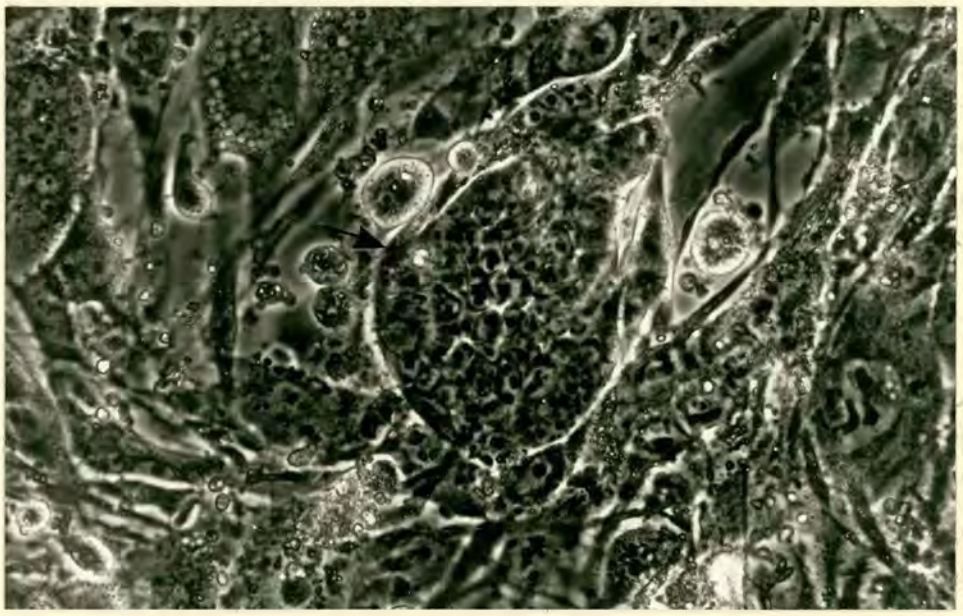
The majority of colonies arising from the disaggregated pieces of ICM were of a differentiative morphology. Trophoblast and endodermal colonies were the most common (figure 2.2a), however, patches of fibroblast-like, neuronal and with extended culture, beating muscle, were also observed. Undifferentiated cells possessing an ES-like morphology (so-called at this stage, since many of the colonies initially displaying this cellular morphology had differentiated by the second or third passage) were identified as small, rounded cells with a large nuclear to cytoplasmic ratio and containing one or more prominent nucleoli (figure 2.2b). On the STO feeders, the ES cells grew in tightly-packed, three-dimensional colonies, where clear intercellular boundaries were difficult to distinguish (figure 2.2c). The first passage was used to screen out all of the differentiated colonies and select only those displaying a stable ES-like cell morphology. This was achieved by employing a relatively long culture interval of five to eight days, feeding the cultures initially every other day, but more frequently whenever the media became acidic.

Figure 2.2: Morphology of cell colonies following disaggregation of ICM outgrowths. Giant trophoblast cells (T) and endodermal-like cells (E) were the most common cell colonies present in first passage cultures of disaggregated ICMs (a). However, some small ES-like colonies were present amongst the assortment of differentiated cells (a) (ES-like colonies are arrowed in all panels). Higher magnification of a small ES-like colony two days after the first passage (b) shows the typical undifferentiated cell morphology. ES cells grew in close opposition with one another, with each cell having a high nuclear to cytoplasmic ratio and one or more prominent nucleoli (b). After seven days of culture on the STO feeders, some cells of the disaggregated ICM had grown into tightly-packed, three-dimensional ES-like colonies, where clear intercellular boundaries were difficult to distinguish (c). The colony shown in panel (c) was subsequently expanded and maintained as a permanent ES cell line.

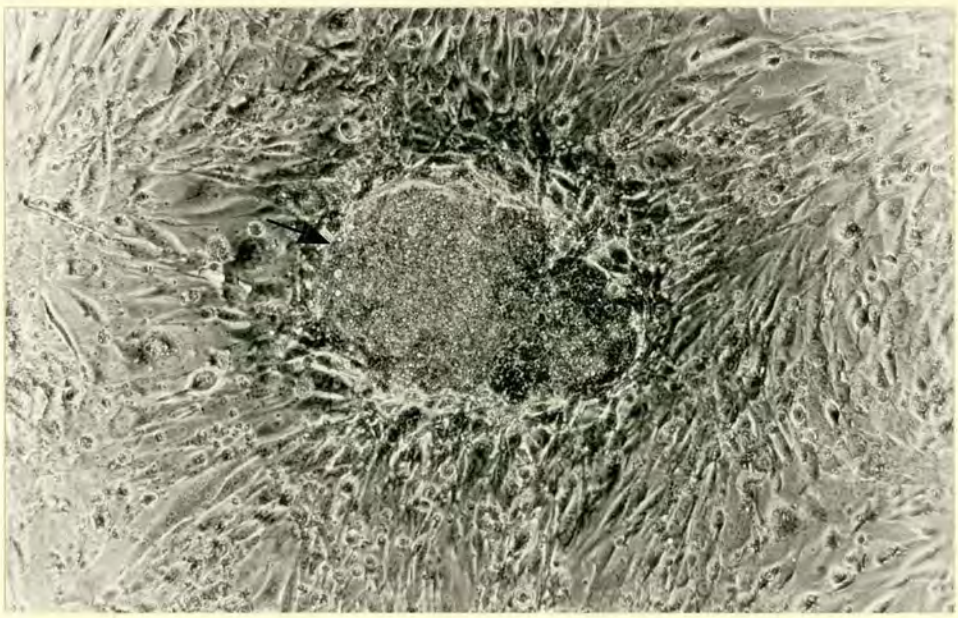
Photographs were taken using phase contrast microscopy.
Magnification: (a,c) x100 and (b) x200

a

b



c



2.3.2 Expansion of Embryonic Stem Cells

The first passage cultures were monitored daily and disaggregated once ES-like cell colonies had attained a "suitably large size", without having commenced differentiation (*e.g.* figure 2.2c). Because not all of the colonies derived from each embryo were of a similar size, the approach to disaggregation at the second passage depended upon the number of stably growing colonies in each culture. If several ES-like colonies were present in the first passage microdrop, then the entire microdrop was trypsinised. The old medium was aspirated, the cells washed *in situ* with PBS and 20µl of TEG was introduced. Following three to five minutes at 37°C, the dish was transferred to the dissecting microscope and a fine, mouth-controlled, pipette used to further dissociate the colonies into single-cells. This was important, so as to prevent subsequent differentiation. The cell suspension was then transferred into a STO feeder well of a four-well plate, containing 1ml of ES_{20} medium. After one or two days, small colonies of ES-like cells could be visualised in some of these cultures.

If only one or two ES-like colonies in the first passage culture were ready for disaggregation they were picked out of the microdrop, while any remaining ES-like colonies, which were considered too small to readily survive the trypsinisation procedures, were left to proliferate further. The larger ES-like colonies were picked off the STO feeder layer with a blunt glass probe, washed briefly in TEG and then incubated at 37°C in a fresh drop of TEG for three minutes. After partial digestion, the colonies were disaggregated mechanically into single-cells with a fine pipette and introduced into a fresh STO microdrop, before being expanded into four-well plates, as described above.

From the four-well plates, the ES-like cells were subsequently expanded into 25cm^2 flasks containing a layer of STO feeder cells. The ES-like cells were cultured within the wells for four to five days, feeding as required, by which time the colonies may have merged to form a monolayer of cells. Working in the flow cabinet, the medium within the well was aspirated and the cells were washed with 1ml of PBS. Following a three to five minute incubation in 0.25ml of TEG, a hand-pulled Pasteur pipette (tip diameter *c.a.* 1mm) and bulb were used to firstly introduce around 0.5ml of ES_{20} medium to neutralise the trypsin and then to vigorously pipette the suspension to disaggregate the cells. The single-cell suspension was then transferred into a flask containing 10ml of ES_{20} medium. The flask was then cultured in the CO_2 incubator with either the cap loosened or, tightened after being gassed with a 5% CO_2, 20% O_2 and 75% N_2 mixture.

2.4 CULTURE OF ESTABLISHED MURINE EMBRYONIC STEM CELLS

This section describes the procedures used for the routine maintenance of ES cells, the freezing and thawing of cell lines, the induction of *in vitro* differentiation and the karotype analysis of stem cells.

2.4.1 Maintenance of Embryonic Stem Cell Lines

Cell lines maintaining a stable ES cell morphology were routinely cultured in 25cm^2 flasks. After the fifth passage, ES cells were both weaned from the STO feeder layer and the serum concentration in the medium reduced by half. ES cells were subsequently maintained on gelatin-coated flasks (Bernstein *et al.*, 1973) in medium (ES_{10}; Table 2.1) supplemented with human recombinant DIA/LIF (gift of Dr. J.P. Simons; Department of Molecular Genetics, IAPGR, Roslin) to prevent ES cell differentiation. Although the concentration of the DIA/LIF factor was not determined, a titration assay had shown that the addition of 0.1% (v/v) of the DIA/LIF supernatant to ES_{10} medium was sufficient to minimise ES cell differentiation (Dr. J.P. Simons, *pers. comm.*).

Established ES cell lines were passaged every three to four days, changing the medium every other day, or whenever it became acidic. When confluent, the cells were re-fed two to three hours before the passage, to maximise the plating efficiency. The medium was then aspirated and the cells washed once in PBS. After removal of the PBS, 2ml of TEG was added and the flask incubated at 37°C for three minutes. The extent of trypsinisation was periodically monitored under the inverted microscope. To minimise spontaneous differentiation of ES cells, the aim was to produce a single-cell suspension. To achieve this, it was sometimes necessary to knock the flask against the bench. Once the cells had been dissociated, the trypsin was neutralised with 3ml of ES_{10} + DIA/LIF medium and the suspension mixed thoroughly. Routinely, the cells were sub-cultured by transferring a 1/10th aliquot of this suspension (*i.e.* 0.5ml) into a pre-gelatinised flask (0.1% (w/v) gelatin for around one hour) containing 10ml of fresh ES_{10} + DIA/LIF medium. The volume of the aliquot dispensed was varied depending on the requirement for ES cells, however, it was not reduced below a one in 10 ratio.

2.4.2 Freezing and Thawing of Embryonic Stem Cells

Generally, three ampoules of each ES cell line were frozen at a low passage number (between four and seven) and stored under liquid nitrogen. The cryoprotectant used in the medium was dimethyl sulphoxide (DMSO; Sigma) at a final concentration of 10%. Although the procedures described here (based on Robertson, 1987) are specifically for ES cells, similar methods were used for the freezing and thawing of STO cells.

2.4.2.1 Freezing Cells

The ES cells from one confluent $25cm^2$ flask (*c.a.* 1×10^7 cells) were frozen in a 1.0ml cryotube (Nunc; Life Technologies), by firstly harvesting the cells by trypsinisation (section 2.4.1). The cells were then pelleted in a conical, plastic universal (Sterilin) by centrifugation at 1000 r.p.m. for five minutes. The supernatant was aspirated and the cells resuspended in 0.5ml ES_{10} + DIA/LIF medium. The final volume (*i.e.* 1.0ml) was made up with 0.5ml of the freezing medium (20% (v/v) DMSO in ES_{10} + DIA/LIF medium) which was added slowly, while gently flicking the universal. The 1.0ml suspension was dispensed into the cryotube, which was then wrapped in two layers of paper tissue before being placed in the -70°C freezer overnight. For long-term storage, the cryotubes were clipped onto freezing canes and plunged into liquid nitrogen.

2.4.2.2 Thawing Cells

Once retrieved from the liquid nitrogen, the cryotube was thawed quickly in a 37°C waterbath, until the ice crystals had all melted. The cryotube was sterilised by wiping with 70% alcohol. Working at room temperature within the flow cabinet, a sterile Pasteur pipette and bulb were used to transfer the cell suspension in the cryotube into 9ml of ES_{10} + DIA/LIF medium in a conical universal. The cells were then pelleted at 1000 r.p.m. for five minutes. The supernatant was aspirated and the cells were resuspended in 10ml of ES_{10} + DIA/LIF medium and transferred to a pre-gelatinised, $25cm^2$ tissue-culture flask and cultured in the CO_2 incubator. The medium was changed after approximately six hours to remove any cell debris. By seeding the thawed cells at high density, the flasks reached confluence within one to two days.

2.4.3 Induction of *In Vitro* Differentiation in Embryonic Stem Cells

ES cells were induced to form cystic embryoid bodies by the suspension culture of cellular aggregates. Cystic embryoid bodies comprised of an inner layer of ectodermal-like cells, with a Reichert's membrane separating a presumed outer layer of parietal endodermal cells.

Suspension culture was conducted in 6cm tissue-culture dishes (Costar) coated with agarose, as described by Handyside and co-workers (1989); rather than in bacteriological dishes. Two layers of agarose of different concentrations were used. The base layer consisted of 2% (w/v) agarose (Type 1; Sigma) in PBS (Table 2.2) which was dissolved by autoclaving. Approximately, 1.5ml was added per 6cm dish to give an even layer and was left to set at room temperature. A second, thin layer was applied using 1% (w/v) agarose in PBS. Once this had set, 5ml of $DMEM_{10}$ medium + 0.1mM ß-mercaptoethanol (see section 2.1.3 and Table 2.1) was added to each dish and incubated, to allow equilibration. Before use, the medium was replaced.

ES cells grown on gelatin-coated flasks were lightly trypsinised with 2ml TEG for one minute. Gentle rocking detached large clumps of cells and the trypsin was neutralised immediately with $DMEM_{10}$ medium + ß-mercaptoethanol. Approximately, a 1/20 aliquot of this aggregate suspension was dispensed into each agarose-coated dish. At higher seeding densities, the individual aggregates adhered to each other. Cultures were fed regularly, aspirating the old medium by either transferring the suspension firstly into a conical universal to allow the embryoid bodies to settle, or by simply tilting the dish, before fresh medium was added. Utilising these procedures, cystic embryoid bodies generally formed within seven to 10 days.

2.4.4 Karyotype Analysis of Embryonic Stem Cells

Metaphase spreads were prepared essentially as described by Robertson (1987) except that the cultures were not exposed to colcemid. Instead, $25cm^2$ flasks of ES cells were taken in an exponential phase of growth, in order to maximise the number of cells in mitosis. ES cells were trypsinised and pelleted in a 20ml conical universal as described previously (section 2.4.2.1). The medium was aspirated and the cell pellet disrupted, before using a Pasteur pipette and bulb to introduce dropwise, around 0.5ml of a 0.56% (w/v) KCl solution. Once mixed, excess hypotonic KCl solution was added to make 10ml and was left at room temperature for 15 minutes, to allow the cells to swell. Following centrifugation (1000 r.p.m. for five minutes) and

aspiration of the supernatant, freshly prepared fixative (three volumes of absolute methanol to one volume of glacial acetic acid) was slowly added dropwise, whilst flicking the tube. Excess fixative was added to make 10ml and the cells were left for five minutes, at room temperature, before pelleting (1000 r.p.m. for five minutes) and aspiration of the supernatant. This cycle was repeated two further times, each time with freshly prepared fixative. The cells were finally suspended in around 0.5ml of fixative and were then ready for producing mitotic spreads.

Metaphase spreads of the fixed, swollen cells were made on clean, wet, glass microscope slides that had been chilled on ice. A quantity of the fixed suspension was drawn into a hand-pulled Pasteur pipette (tip diameter *c.a.* 1mm) with a bulb. Several drops of suspension were released onto the slide from a height of around 100cm. The undersurface of the slide was wiped dry and evaporation of the fixative was aided by warming the slide briefly in the flame of a Bunsen burner, as well as blowing across the surface of the slide. The height from which the cells fell and the rate of fixative evaporation, were both important variables in maximising the rupture of the swollen cells and hence, the spreading of the chromosomes. At least five slides per ES cell line were prepared in this manner. The slides were stored in a dust-free drawer for 10-14 days before G-banding.

Slides were incubated in 2X standard saline citrate (SSC: 0.3M NaCl, 0.03M trisodium citrate) at 60°C for one hour. The slides were then rinsed four to five times in distilled water and stored temporarily in a rack under water. Each slide was individually immersed in a 0.25% (w/v) trypsin (1:250 Difco; Difco Laboratories) solution in Gurr's phosphate buffer (pH 6.8; BDH) for between 7-15 seconds at room temperature. The trypsin remaining on the slide after this digestion, was neutralised in Gurr's buffer containing 5% (v/v) NCS. The slides were further rinsed in two changes of buffer before being stained in freshly prepared 5% (v/v) Giemsa Gurr's R-66 stain (BDH) in Gurr's buffer (pH 6.8) for 8-10 minutes. Slides were finally rinsed in two changes of buffer, followed by two changes of distilled water and allowed to air dry.

G-banded metaphase spreads were examined with a standard format, brightfield microscope utilising oil immersion, objective lenses (maximum magnification: x1000; Olympus BH-2). Chromosome numbers were counted and the sex of the cell line determined from at least 10 metaphase spreads prepared from cells between culture passages six and 15. Suitable spreads were photographed on Technical Pan, 25 ASA film (Kodak). G-banded chromosomes were identified according to the Standardised Genetic Nomenclature for mice (Nesbitt and Francke, 1973).

2.5 PRODUCTION OF CHIMAERIC MICE

The *in vivo* pluripotency of isolated ES cell lines was tested by the production of chimaeric mice. This was achieved by utilising a micromanipulative technique, employing two micropipettes, to physically inject the ES cells into blastocyst-stage embryos in a method similar to that described by Bradley (1987).

2.5.1 Donor and Recipient Mice

Donor, day 3.5 *p.c.* blastocysts were obtained from albino, MF1 strain females mated to MF1 stud males. Procedures for superovulation (section 2.2.1) and embryo recovery (section 2.2.2) have been described previously. Following recovery, embryos were group-cultured in a drop of ES_{10} + DIA/LIF medium under oil, until they were required for manipulation.

Pseudo-pregnant females were required to act as recipients for the surgically transferred, manipulated embryos. Recipients were obtained from natural matings between MF1 or F_1 (C57BL/6 X CBA/Ca) females and vasectomised males. The approximate time of ovulation was determined by checking for copulation plugs the following morning, which was designated day 0.5 *p.c.*. These matings were set up so that the recipient females were one day less advanced (*i.e.* 2.5 days *p.c.*) than the donor embryos.

2.5.2 Preparation of Embryonic Stem Cells

ES cells for blastocyst injection were maintained on gelatin-coated flasks in ES_{10} + DIA/LIF medium as outlined in section 2.4.1. To maximise stem cell viability, the medium was changed every day and the cultures fed two to three hours prior to trypsinisation. The cells were cultured on a three day passaging regime, with ES cells being used for injection on days one or two of the growth cycle. The cells were trypsinised into a single-cell suspension and a small volume, containing several hundred cells, was introduced into the injection chamber (see section 2.5.4) on the microscope stage. After one to two hours of micromanipulation, these cells were discarded and a new injection chamber was prepared with freshly trypsinised ES cells.

2.5.3 Preparation of Micropipettes

Two micropipettes, constructed from glass capillary tubing, were utilised for the embryo manipulations. The blastocyst was immobilised by gentle

suction with a holding pipette and an injection pipette used to introduce the ES cells into the blastocoelic cavity.

The holding pipette was constructed by heating a small section of a 10mm long capillary tube (external diameter 1.0mm; GC100-10, Clark Electromedical Instruments) uniformly over the pilot flame of a propane gas burner. Once softened, the capillary was withdrawn from the flame and pulled quickly by hand, to produce a gradual taper over a 2-3cm length of glass. This pulled capillary was then broken at right angles on a microforge (Research Instruments Ltd.) at an external diameter of 90-100μm. This was achieved by positioning the capillary vertically on the microforge and placing the glass at the required diameter (determined by a graticular eyepiece) adjacent to the glass bead on the platinum wire, heating filament. With the glass bead heated slightly (the temperature was determined empirically) the capillary was then brought into contact so that the two just barely fused together. The filament was then switched off and at the same instant the spring-loaded connecting rod, holding the filament, was depressed and so, causing the capillary to break cleanly at the point of contact with the glass bead. The tip was then heat-polished by placing the tip above, but just clear of, the hot glass bead, causing the glass to melt and produce a concentric orifice of 20-25μm in diameter.

The injection pipettes were prepared by pulling thin-walled, 1.0mm diameter glass capillary tubing (GC100T-15; Clark Electromedical Instruments) on a horizontal pipette puller (model 753; Campden Instruments Ltd.). The settings were adjusted to produce a gradual taper over a 15mm length of glass, from the shoulder to the needle point. The microforge was then utilised to break the capillary at right angles at an external diameter of 18-20μm, in a manner similar to that described above. The tip of the pipette was bevelled to an angle of 45°. This was accomplished by grinding the pipettes for three to five minutes each, on a slowly rotating (60 r.p.m.) aluminium oxide disc (1μm grade; 3M) lubricated with distilled water and with a stream of air flowing through the inside of the pipette, to keep the internal bore free of debris. With the aid of the microforge, a sharp point was put onto the leading edge of the bevelled pipette. With the pipette positioned vertically, the filament was heated to a low temperature, at which the pipette tip just barely fused to the glass bead. Once fused, the pipette was raised away from the filament to draw out a short glass spike. To provide a "siliconising" effect, the tip of the pipettes were washed in a 1.25% (v/v) detergent solution of Tween-80 (Sigma) in distilled water and allowed to dry at room temperature, before use.

Using the microforge, both the injection and holding pipettes were bent at an angle of around 30°, 5mm from the respective tips, to allow entry into the injection chamber (see figure 2.3).

2.5.4 Micromanipulator Assembly

The injections were performed using two Leitz micromanipulators mounted on either side of a fixed-stage, inverted, phase contrast microscope (magnification: x40, x100; Nikon Diaphot). The two instrument holders were each connected to micrometer syringes (Hamilton) using thick-walled (Portex) tubing (internal diameter 2.5mm) filled with inert mineral oil (Fluorinert 77; Sigma). The holding pipette was held in the left-hand manipulator, with the corresponding (500µl) micrometer syringe positioned on the right-hand side. The reverse arrangement was established for the injection pipette and the associated (250µl) micrometer syringe. Before fixing the pipettes into the respective tool holders, the tubing was purged of air bubbles using a 20ml syringe filled with Fluorinert, which was connected into the hydraulic line via a three-way tap. The pipettes were locked into the tool holders and using the two reservoir syringes, each pipette was filled with Fluorinert. The pipettes were then aligned in the injection chamber so that the ends were parallel to the bottom surface (figure 2.3). Medium from the chamber was then drawn a short distance into the pipette using the reservoir syringes. The injection chamber was prepared by introducing a 250µl drop of ES_{10} + DIA/LIF + 20mM HEPES buffered medium, in the centre of a 90mm bacteriological dish (Sterilin) which was overlayered with paraffin oil. Several hundred ES cells were then introduced into the chamber and the manipulations were performed at room temperature.

2.5.5 The Blastocyst Injection Procedure

Groups of five embryos were transferred from the incubator into the injection chamber. The injection pipette was lowered to the base of the chamber and ES cells for one injection were individually selected and gently drawn into the pipette, one behind the other. The expanded blastocysts were held by suction onto the holding pipette. By patiently blowing and sucking medium, through the holding pipette, the embryo was eventually orientated in the desired position, with the ICM region over the orifice of the pipette (figure 2.3). Focusing, at x100 magnification, on a "junction" between where two trophectodermal cells joined together, around the equatorial plane of the embryo, the injection pipette was then raised to the same focal level. The injection pipette was then brought to a point just adjacent to this cellular junction (figure 2.3). With a rapid movement forward on the manipulation joystick, the pipette was pushed into the blastocoelic cavity. The ES cells were gently blown out of the injection pipette and into the embryo. Between 10 and 15 healthy ES cells were introduced. The injection pipette was carefully withdrawn from the blastocyst

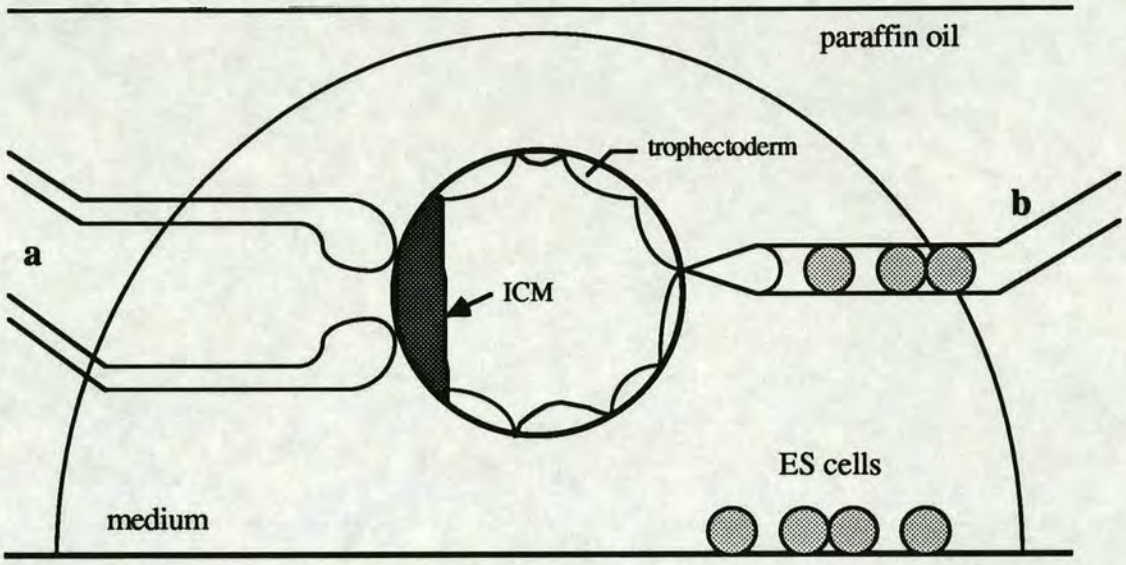


Figure 2.3: Orientation of micropipettes within the manipulation chamber. A holding pipette (a) was used to immobilise the blastocyst by gentle suction, while an injection pipette (b) was used to introduce the ES cells into the blastocoelic cavity of the embryo.

and the injected blastocyst was released from the tip of the holding pipette. Shortly thereafter, the blastocysts generally collapsed due to the punctured trophectoderm. They were then transferred to the incubator in a droplet of ES_{10} + LIF medium under oil, where they slowly began to re-expand. Injected embryos were cultured for between 30 minutes and three hours before being transferred to recipients, regardless of whether or not the blastocysts had fully re-expanded.

2.5.6 Embryo Transfer

The injected blastocysts were surgically transferred into the uterine lumen of day 2.5 *p.c.* pseudo-pregnant recipients. Typically, 10 embryos were transferred unilaterally. Surgical operations were conducted in a laboratory within the mouse house. Thus, females did not leave the facility and were returned the the same cage-rack after the transfer. This minimised animal stress and losses due to embryonic resorptions.

Recipients were anaesthetised with a 0.15 to 0.20ml intraperitoneal injection of a Hypnorm (Janssen Pharmaceuticals) and Hypnovel mixture (Roche Products). This anaesthetic was prepared by mixing separately, 1ml of Hypnorm (containing 0.315mg fentanyl citrate and 10mg fluanisone) and 1ml of Hypnovel

(containing 5mg midazolam hydrochloride) each with 2ml of distilled water, **before** combining the two solutions together. The anaesthetic was stored at room temperature and replaced after four weeks.

Once unconscious, the back of the mouse was wiped with 70% alcohol. A pair of blunt forceps were used to pick up the skin and dressing scissors were used to make a 10mm lateral incision across the midline, posterior to the last rib. Paper tissues, moistened with 70% alcohol, were used to open the wound and remove any cut hair, by wiping in a head to tail direction. Sliding the skin from side to side, the position of the right-hand ovary was located beneath the body wall. The region directly overlying the ovary was grasped with pointed watchmakers forceps and a 5mm incision was made in the body wall with sharp iris scissors. By exerting gentle pressure on both sides of the incision, the ovarian fat pad was exteriorised and grasped with blunt forceps. Very carefully, the associated ovary, oviduct and the top of the uterine horn were also pulled out. The weight of the fat pad was generally sufficient to keep the ovary from slipping back inside the abdominal cavity. The mouse was then transferred, on absorbant tissues, to the stage of a binocular dissecting microscope (Olympus SZH), with a fibre-optic incident light source (Schott, KL1500).

In another dissecting microscope (Olympus SZ), with transmitted illumination, the embryos to be transferred were aspirated into a hand-pulled Pasteur pipette, with an internal diameter just larger than the embryos themselves and with a square tip. The pipette was pre-loaded with alternate media and air bubbles, which allowed fine control over the movement of the embryos. Embryos were drawn up into the pipette closely one behind the other and so, the minimum of medium was introduced into the uterus when the embryos were transferred.

Focusing on the region of the uterotubal junction (magnification *c.a.* x10), a pair of watchmakers forceps grasped the top of the uterine horn gently and lifted it up slightly to allow a 25-gauge hypodermic needle to puncture the uterine wall and enter the lumen. Whilst still holding the top of the uterus and observing down the microscope, the transfer pipette containing the embryos was picked up and the tip inserted 5mm inside the uterine lumen, through the hole produced with the needle. The embryos were gently blown into the uterine lumen using the air bubbles along the pipette as markers. After transfer, the pipette was checked to ensure that all of the embryos had been expelled.

The reproductive organs were returned to the abdominal cavity by lifting up one edge of the incision and pushing in the ovarian fat pad with blunt forceps. The incision in the body wall was closed with a single suture (5/0 Mersilk; Ethicon), while three sutures were used to close the skin. Mice were allowed to

recover on a heated blanket before they were returned to the cage-rack. If pregnant, they littered 17 days later.

2.5.7 Analysis of Chimaeras

All of the ES cell lines isolated in these studies were of a 129/Sv-*CP* or F_2 (C57BL/6 X CBA/Ca) genotype; that is, they possessed homozygous dominant, pigmented coat colour alleles. The 129/Sv-*CP* strain used here has a black agouti (BB AA) phenotype, whereas the F_2 embryos, produced by crossing F_1 [C57BL/6 (black, non-agouti: BB aa) X CBA/Ca (black agouti: BB AA)] parents, will segregate into both black agouti (AA, Aa) and black non-agouti (aa) phenotypes in the ratio of 3:1. As donor blastocysts were obtained from the homozygous recessive, albino (c/c) MF1 strain, it was a simple matter to assess chimaerism and germline transmission of the ES cells on the basis of coat colour markers.

Overt chimaeras, in some instances, were determined at birth from eye colour or three to four days later, by the presence of pigment in the skin. An estimate of the percentage contribution of ES cells to the skin was also taken. The sex of the chimaeras was assessed at weaning; at three to four weeks of age.

With XY ES cell lines, male chimaeras were backcrossed by mating to MF1 (albino) females to assay for germline transmission. This was detected by the presence of pups with an entirely pigmented coat, from the dominant alleles carried by the cultured stem cells. With XX ES cell lines, female chimaeras were mated to MF1 males to test for ES cell-derived oocytes.

2.6 STATISTICAL ANALYSES

In this thesis, murine ES cell lines are defined as cell lines from individual embryos which maintained a stable ES cell morphology for at least 10 passages in culture and possessed the capacity to form cystic embryoid bodies following *in vitro* differentiation. During preliminary studies, it was found that in the tissue-culture facility utilised throughout this thesis, colonies derived from day 3.5 *p.c.* mouse embryos from the 129/Sv-*CP* strain and identified as possessing an ES cell morphology at the third passage, were subsequently expanded into permanent ES cell lines. Thus, in the experimental studies reported in this thesis, ES cell colonies (derived from 129/Sv-*CP* blastocysts) which had been stably maintained up to at least the third passage but which were subsequently lost to rare cases of infection, were still classed as ES cell lines. Any embryos or cultures which were lost to infection before the third passage were eliminated from the data. During the initial phases of

establishment of ES cell lines, between the disaggregation of the ICM and before passage three, colonies possessing a stem cell morphology are referred to as "ES-like" to signify the fact that many of these colonies were morphologically unstable at these early stages of culture.

In studying the factors which influence the capacity of embryos to yield ES cell lines, the data are presented in two formats in this thesis. Firstly, as the efficiency of ES cell isolation from a particular experimental treatment; expressed as the percentage of the number of embryos yielding an ES cell line divided by the total number of embryos cultured. Secondly, the results are also presented as "survival profiles" showing the proportion of embryos giving rise to ES-like colonies at each successive passage *in vitro*, until the establishment of permanent ES cell lines. Only colonies which maintained a stable ES-like cell morphology throughout each passage were included in the data. The survival profiles were expressed percentages, at each passage, of the number of embryos yielding ES-like colonies divided by the total number of embryos cultured for each treatment.

Most experiments were repeated with a number of different batches of embryos, cultured on different occasions. These served as replicates of the experiment from which the standard error of the means (s.e.m.) were calculated, according to the following formulae:

$$S = \sqrt{\sum_{i=1}^{n} (X_i - \bar{X})^2 / (n - 1)}$$

$$\text{s.e.m.} = \frac{S}{\sqrt{n}}$$

Where: S = sample standard deviation
Σ = summation
n = number of samples
i = the i_{th} sample
X = sample value
$\bar{X}$ = sample mean (from: Snedecor and Cochran, 1980)

The statistical analyses of the data were accomplished with the chi-squared test, comparing two proportions. The proportions were arranged initially in a four-fold table as illustrated below (*e.g.* comparing the efficiency of ES cell isolation between treatments a and b):

	Treatments		
	a	b	Total
Number of embryos giving ES cell lines	n_{11}	n_{12}	$n_{1.}$
Number of embryos giving only differentiated colonies	n_{21}	n_{22}	$n_{2.}$
Total number of embryos cultured	$n_{.1}$	$n_{.2}$	$n_{..}$

(from: Fleiss, 1973)

From the above fourfold table, the magnitude of the chi-squared (χ^2) statistic was calculated according to the formula:

$$\chi^2 = \frac{n_{..}\,(|n_{11}n_{22} - n_{12}n_{21}| - 0.5n_{..})^2}{n_{1.}n_{2.}n_{.1}n_{.2}}$$

(from: Fleiss, 1973)

When the chi-squared statistic exceeded 3.84, a statistically significant difference (at the $P<0.05$ level) between the two proportions was said to exist.

CHAPTER 3

INFLUENCE OF MOUSE GENOTYPE AND IMPLANTATIONAL DELAY ON THE EFFICIENCY OF EMBRYONIC STEM CELL ISOLATION

3.1 INTRODUCTION

There are limited reports in the literature quantifying the potential of embryos from different mouse strains to generate ES cell lines. While ES cells have been isolated from a wide range of genetic backgrounds (see chapter 1.3.3.2) it is not clear whether some mouse genotypes are more "permissive" than others. The effect of mouse genotype has been confused as many workers have utilised a variety of crossbred embryos for ES cell isolation. Furthermore, despite the use of similar isolation methods, there have been some conflicting reports on the effect of mouse genotype; perhaps highlighting the influences of unknown variables in the tissue-culture conditions between different laboratories. In a study primarily examining the effect of feeder cell layers (Suemori and Nakatsuji, 1987) there was no difference in the proportion of embryos from the 129 (/J and /SvJ) and C57BL/6 inbred strains giving rise to ES cell lines (both 18%; 36/201 and 30/167, respectively). Doetschman and associates (1985), however, reported that embryos from the C57BL/6 strain generated ES cell lines at twice the frequency (10%) as embryos from the 129/Sv strain (5%), although data on the numbers of embryos cultured and the significance of these results were not reported. Although no specific mouse strains were mentioned, one study has reported that mouse strains could be classed as possessing either a high or a low potential to give rise to ES cells in culture (Martin *et al.*, 1984). Subsequently, it was reported by this group that mutations at the mouse *t*-locus have significant effects on the frequency at which ES cells can be established. The ICM from embryos homozygous for the t^0 haplotype are unable to proliferate *in vitro* and give rise to ES cells (Martin *et al.*, 1987). A laboratory experienced in isolating ES cells from a range of mouse strains has not, however, published the associated efficiencies (Robertson *et al.*, 1983b). This group have reported that none of the strains they examined could be classed as "non-permissive" and routinely expect

approximately 10% of all day 3.5 *p.c.* blastocyst-stage embryos explanted into culture to yield ES cell lines (Robertson and Bradley, 1986).

Female mice have a facultative form of implantational delay, one which is imposed by lactation (Bergstrom, 1978). Implantational delay may also be induced experimentally in females three days after mating, by surgical removal of ovarian tissue, in combination with post-operative administration of a synthetic progesterone (Bergstrom, 1978). The embryos progress to the hatched blastocyst-stage and although they remain viable, their development thereafter is arrested as a consequence of these procedures. It has been reported that "delayed" embryos, produced experimentally, have a greater capacity to yield ES cell lines than non-delayed, day 3.5 *p.c.* embryos (Robertson and Bradley, 1986). The mechanism responsible for this effect of implantational delay is not understood.

The 129/Sv-*CP* mouse strain used in these studies was similar to that originally employed by Evans and Kaufman (129/Sv//Ev: 1981). The 129 strain has not been demonstrated to yield ES cell lines at a significantly higher efficiency compared to other mouse genotypes. However, it is a strain that is commonly favoured for ES cell isolation and lines derived from the 129 strain have been utilised extensively for gene targeting experiments (reviewed by Baribault and Kemler, 1989).

In preliminary experiments, the isolation of ES cells from crossbred F_2 (C57BL/6 X CBA/Ca) embryos had proved very difficult, compared to embryos from the inbred 129/Sv-*CP* strain. The objective of this study was to investigate whether a significant difference existed between the potential of embryos from these two mouse genotypes to yield ES cells, in addition to examining the effect of imposing an artificial period of diapause on the embryos from these two genotypes. This study validated the reliability of the culture methods utilised in this thesis.

3.2 EXPERIMENTAL METHODS

The efficiency of ES cell isolation from two mouse genotypes was compared between normal day 3.5 *p.c.* blastocysts and blastocysts that had undergone a three or five day period of implantational delay. Embryos were recovered from both 129/Sv-*CP* females mated to 129/Sv-*CP* males (*i.e.* 129/Sv-*CP* embryos) and from F_1 (C57BL/6 X CBA/Ca) females mated to F_1 (C57BL/6 X CBA/Ca) males (*i.e.* F_2 embryos). The general procedures utilised for the superovulation of females and the isolation, maintenance and manipulation of ES cells were the same as those described in chapter two.

3.2.1 Production of Implantationally Delayed Blastocysts

Ovariectomy was performed on superovulated females 2.5 days *p.c.* (Bergstrom, 1978). The procedures for anaesthesia and the exteriorisation of the ovary from the abdominal cavity were the same as those described previously (see chapter 2.5.6). The mouse was then transferred, on absorbent tissues, to the stage of a binocular dissecting microscope (Olympus SZH), with a fibre-optic incident light source (Schott, KL 1500). Focusing on the ovary at low magnification (*c.a.* x10), two watchmakers forceps were utilised to delicately tear open the ovarian bursa, encapsulating the ovary and the oviduct. With the ovary released, a small, pre-tied loop (*c.a.* 5mm diameter) of fine suture cotton (5/0 Mersilk; Ethicon) was slipped between the ovary and the oviduct and pulled tight with forceps, to ligate the ovarian blood vessels. All of the ovarian tissue was then cut away, using iris scissors.

The fat pad and oviduct were replaced inside the abdominal cavity by lifting up one edge of the incision and pushing in the fat pad with blunt forceps. The ovariectomy was then repeated on the other side. The incisions in the body wall were closed with single sutures (5/0 Mersilk; Ethicon) while the skin was closed with three sutures.

While the female was still unconscious, 0.1ml of a 10mg/ml progesterone (4-pregene-3, 20-dione; Sigma) solution, dissolved in absolute alcohol, was injected subcutaneously into the flank region. The mouse was placed onto a heated blanket to aid post-operative recovery.

The delayed blastocysts were recovered from the uterine horns as outlined in chapter two (section 2.2.2). Embryos from the 129/Sv-*CP* strain were flushed on days 6.5 and 8.5 *p.c.* (*i.e.* four and six days after ovariectomy; or, three and five days, respectively, after blastocyst formation) and F_2 (C57BL/6 X CBA/Ca) embryos were recovered 8.5 days *p.c.*.

3.3 RESULTS

3.3.1 Embryo Recovery

The average yield of embryos from superovulated 129/Sv-*CP* females recovered on day 3.5 *p.c.* was 7.8 ± 0.9 (n = 18). The ovariectomy procedure, utilised to produce implantationally delayed blastocysts, did not alter the recovery of 129/Sv-*CP* embryos when the uterine tracts were flushed 6.5 days *p.c.* (8.4 ± 1.5; n = 11). However, when other 129/Sv-*CP* females from the same batch of ovariectomised mice were flushed two days later (*i.e.* 8.5 days *p.c.*) the number of

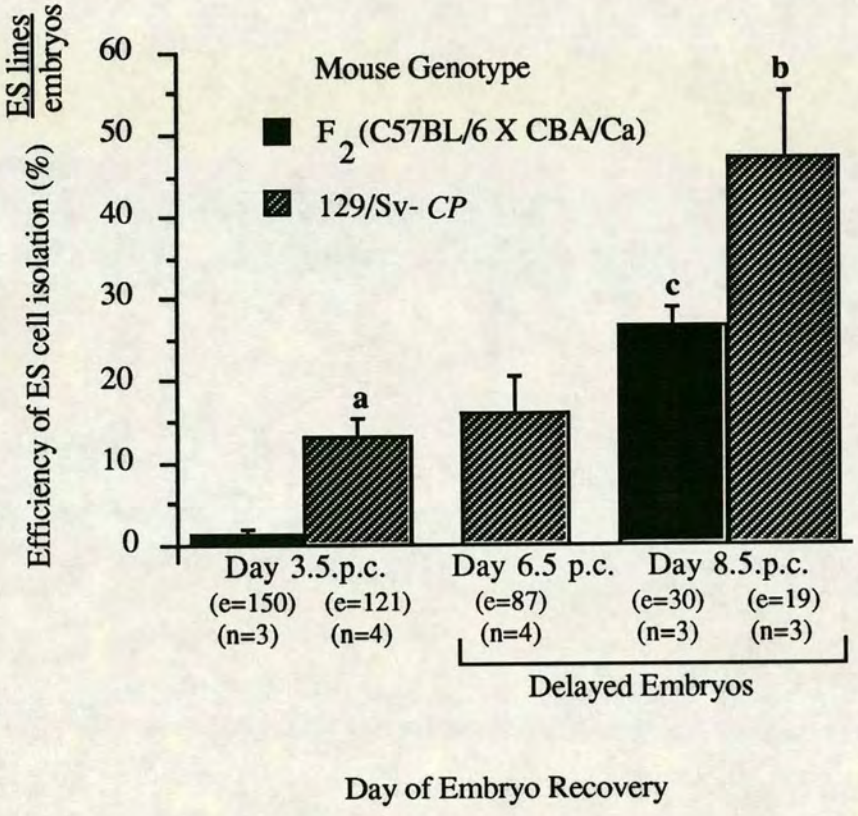


Figure 3.1: The effects of mouse genotype and implantational delay on the efficiency of ES cell isolation. (mean ± s.e.m.; e = embryos; n = replicates).

a = P<0.001 compared to day 3.5 *p.c.* F_2 embryos.

b = P<0.001 compared to day 3.5 *p.c.* 129/Sv-*CP* embryos.

b = P<0.01 compared to day 6.5 *p.c.* 129/Sv-*CP* delayed embryos.

c = P<0.001 compared to day 3.5 *p.c.* F_2 embryos.

embryos recovered (1.9 ± 0.6; n = 10) was significantly less (P<0.001). This effect of the period of delay, was not observed in the recovery of F_2 (C57BL/6 X CBA/Ca) embryos; where the yield was not different between day 3.5 *p.c.* (14.0 ± 3.0; n = 12) and ovariectomised F_1 females, flushed on day 8.5 *p.c.* (10.0 ± 0.6; n = 3).

3.3.2 The Effects of Mouse Genotype and Implantational Delay on the Efficiency of Embryonic Stem Cell Isolation

Significant and reproducible trends in the effects of mouse genotype and implantational delay on the efficiency of ES cell isolation were observed (figure 3.1). The standard error of the means in figure 3.1, reflected the slight variability between the different batches of embryos cultured throughout the course of this experiment; each treatment was repeated either three or four times.

Day 3.5 *p.c.* blastocysts from the 129/Sv-*CP* mouse strain yielded significantly (P<0.001) more ES cell lines (16/121=13.2 ± 2.3%) than embryos of the F_2 (C57BL/6 X CBA/Ca) genotype (2/150=1.3 ± 0.6%; figure 3.1). The effect of implantational delay on the efficiency of ES cell isolation, depended upon the time the blastocysts remained dormant within the uterus. When "delayed" embryos of the 129/Sv-*CP* mouse strain were cultured following recovery on day 6.5 *p.c.*, there was no significant difference in the yield of ES cell lines (14/87=16.1 ± 4.4%) compared to day 3.5 *p.c.* embryos. Extending the period of implantational delay of blastocysts to five days, however, (*i.e.* embryo recovery was on day 8.5 *p.c.*) significantly increased the capacity of the 129/Sv-*CP* strain embryos to give rise to ES cell lines (9/19=47.4 ± 7.8%) compared to both day 3.5 *p.c.* embryos (P<0.001) and delayed embryos recovered on day 6.5 *p.c.* (P<0.01). Similarly with the F_2 embryos, a significant increase was observed with delayed blastocysts recovered 8.5 days *p.c.* (8/30=26.7 ± 2.3%; P<0.001) compared to non-delayed, day 3.5 *p.c.* F_2 embryos (figure 3.1). Furthermore, the proportional increase in the efficiency of ES cell isolation resulting from embryonic delay (with embryo recovery on day 8.5 *p.c.*) was significantly (P<0.01) greater with F_2 embryos, compared to the 129/Sv-*CP* embryos (figure 3.1).

The percentage survival of ES-like colonies derived from either delayed (day 8.5 *p.c.* only) or non-delayed (day 3.5 *p.c.*) embryos, of both 129/Sv-*CP* and F_2 (C57BL/6 X CBA/Ca) genotypes, after each successive passage in culture, is plotted in figure 3.2. The graph shows the progressive decline at each passage in the proportion of original embryos giving rise to colonies of ES-like morphology, until the eventual establishment into culture of stable ES cell lines. The loss of some ES-like colonies after each passage was due to either differentiation, with the colonies

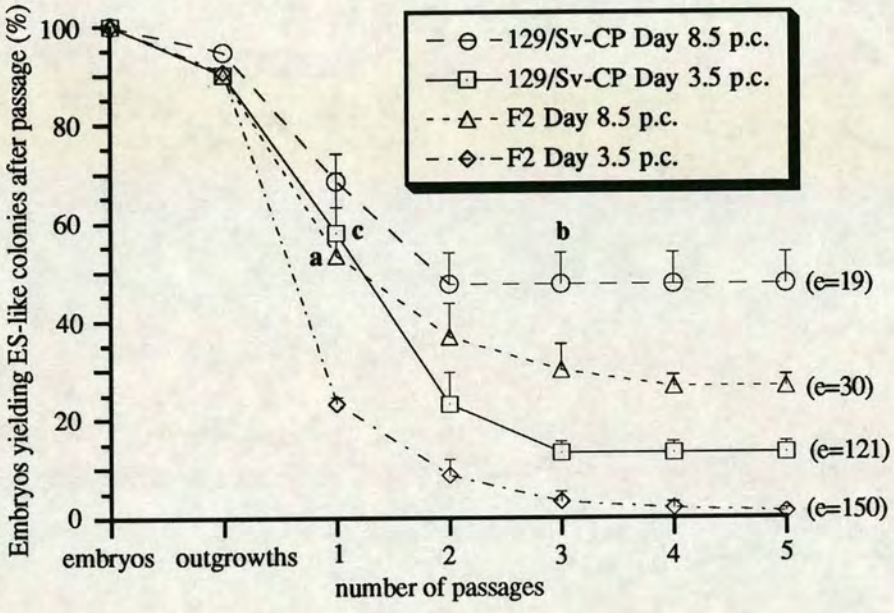


Figure 3.2: The survival profiles showing the effects of mouse genotype and implantational delay on the percentage of individual embryos giving rise to colonies with an ES-like morphology after repeated passage in culture. After passage five, ES cells derived from both 129/Sv-*CP* and F_2 (C57BL/6 X CBA/Ca) embryos were maintained as permanent ES cell lines. (mean ± s.e.m.; e = embryos).

a = P<0.001 compared to the percentage of non-delayed day 3.5 *p.c.* F_2 embryos yielding first passage ES-like colonies.

b = P<0.001 compared to the percentage of non-delayed day 3.5 *p.c.* 129/Sv-*CP* embryos yielding third passage ES-like colonies.

c = P<0.001 compared to the percentage of day 3.5 *p.c.* F_2 embryos yielding first passage ES-like colonies.

displaying an altered cell morphology or, occasionally, to the failure of the cells to survive the trypsinisation protocol.

From figure 3.2, between 5-10% of all the embryos explanted into culture did not produce an outgrowth that was subsequently disaggregated. In the case of day 3.5 *p.c.* expanded blastocysts, this loss was commonly due to failure of the embryos to hatch from the zona pellucida. Instead, some blastocysts collapsed and senesced inside the zona. Occasionally, outgrowths did not possess a progressively growing ICM. Rather, they may have simply flattened early in the process of outgrowth and only formed trophectoderm. Losses were not a consequence of allowing ICM outgrowths to become extensively differentiated, with the formation of endoderm. Explanted day 3.5 *p.c.* embryos, of both mouse genotypes, were disaggregated after three to five days in culture, with the period being extended to four to seven days for delayed embryos. Monitoring outgrowths over these periods allowed each embryo to be disaggregated once it reached an "optimal" size.

With the F_2 embryos, a significantly greater ($P<0.001$) proportion of delayed embryos gave rise to ES-like colonies at the first passage, compared to non-delayed, day 3.5 *p.c.* embryos (16/30=53.3 ± 9.8% *vs* 35/150=23.3 ± 1.3%; figure 3.2). This was a difference that was maintained throughout the subsequent passages. With the 129/Sv-*CP* strain, however, there was no significant difference, at the first passage, in the proportion of embryos yielding putative ES cell colonies between delayed and non-delayed blastocysts (13/19=68.4 ± 5.5% *vs* 70/121=57.9 ± 12.3%). In fact, the difference between delayed and non-delayed 129/Sv-*CP* embryos was not significant until the third passage ($P<0.001$) and resulted from the significantly ($P<0.005$) greater loss of putative ES cell colonies to differentiation with cultures derived from non-delayed day 3.5 *p.c.* embryos (figure 3.2). Comparing day 3.5 *p.c.* embryos from both genotypes, a significantly ($P<0.001$) greater proportion of disaggregated 129/Sv-*CP* embryonic outgrowths gave rise to ES-like colonies at the first passage than F_2 (C57BL/6 X CBA/Ca) outgrowths (57.9 ± 12.3% *vs* 23.3 ± 1.3%, respectively).

At the third passage, colonies derived from 129/Sv-*CP* embryos and identified as having an ES cell morphology, were stably maintained thereafter and expanded into permanent stem cell lines. ES-like colonies from the F_2 (C57BL/6 X CBA/Ca) embryos appeared to be slightly less stable in culture than those from the 129/Sv-*CP* strain (figure 3.2), with one or two "potential cell lines" being lost to differentiation (typically of a trophectodermal lineage) at the fourth and fifth passages. Thereafter, F_2 ES cell colonies were maintained as stable cultures.

Apart from maintaining a stable ES cell phenotype *in vitro*, the other criterion used for defining ES cells was their capacity to form cystic embryoid bodies when cultured in suspension. ES cell lines, classified on the basis of cell morphology, from delayed and non-delayed embryos of both F_2 and 129/Sv-*CP* genotypes, all appeared to be equally capable of differentiation *in vitro*.

3.3.3 Production and Analysis of Chimaeras

The pluripotency of one male ES cell line derived from an F_2 delayed blastocyst (designated $F_2$18) was tested *in vivo*. Approximately 74% (14/19) of the metaphase spreads examined from the $F_2$18 cell line at passage six had an apparently normal 40XY chromosome constitution, with the remainder being pseudodiploid. The stem cells were injected into MF1 strain, host blastocysts between passages eight to 12. Of the manipulated embryos transferred to recipient females, 60% (53/89) survived to term (Table 3.1). Of these pups, 28% (15/53) were overtly chimaeric on the basis of coat colour. The extent of ES cell contribution to the skin of the chimaeras was variable, ranging between estimates of 20-90%. There was a slight shift in the sex ratio amongst the chimaeric population, with two-thirds of the chimaeras being phenotypic males (Table 3.1). Two overt hermaphrodites, with both male and female external gentitalia, were produced in this series of blastocyst injections. The limited test breeding of seven chimaeric males did not demonstrate transmission of the ES cell-derived F_2 (C57BL/6 X CBA/Ca) genotype through the germline, in a total of 226 pups born from 20 litters.

Table 3.1: Production of chimaeras from an embryonic stem cell line derived from an F_2 (C57BL/6 X CBA/Ca) delayed blastocyst.

ES Line	Sex	Blastocysts Injected	Pups Born (%)	Chimaeras (%)	Sex Ratio
$F_2$18	XY	89	53 (60)	15 (28)	10 males 3 females 2 overt hermaphrodites

3.4 DISCUSSION

The findings presented in this chapter have demonstrated the significant effects of mouse genotype and implantational delay on the efficiency of ES cell isolation. The proportion of intact day 3.5 *p.c.* embryos from the 129/Sv-*CP* strain yielding ES cell lines in this study (13.2 ± 2.3%) was comparable to the levels reported previously in experienced hands (Robertson and Bradley, 1986). The F_2 embryos, produced by crossing F_1 (C57BL/6 X CBA/Ca) males and females, possessed a significantly lower potential to give rise to stem cells in this study (1.3 ± 0.6%) compared to 129/Sv-*CP* embryos.

The genetic basis for the difference between the 129/Sv-*CP* and F_2 embryos is not clear. There was no obvious difference in the rate of blastocyst outgrowth between the 129/Sv-*CP* and F_2 embryos, as might have been expected between embryos of different genotypes. Disaggregation of the ICM outgrowths from both strains, was equally distributed over the three to five day period after explanting the embryos into culture. There was no consistent difference in the size of the ICM before the first signs of differentiation, nor any apparent correlation between ICM size and the occurrence of ES-like primary colonies. The significant difference, between the two mouse genotypes, was in the proportion of embryos giving rise to ES-like colonies at the first passage (figure 3.2). The ICM cells of the F_2 embryos appeared to be more predisposed to differentiation *in vitro*, especially to trophectodermal and endodermal cell lineages.

Early studies on the formation of teratocarcinomas, following the transplantation of embryos to extra-uterine sites, have shown differences in the permissiveness of embryos from different strains to teratocarcinogenesis (Solter *et al.*, 1979) and this may be related to the efficiency of ES cell isolation. Although this process depends upon the use of syngeneic or histocompatible F_1 hybrid, graft-receiving host animals, there also appears to be an effect of the genotype of the mouse embryo. Embryos from the 129/Sv strain are highly permissive to teratocarcinogenesis when transplanted to an ectopic site (Stevens, 1970c). Furthermore, the 129/Sv strain are a line of mice in which the males have a high incidence of spontaneous testicular teratoma (Stevens and Little, 1954). The genes Sl^J (Steel), *C* (full pigment) and *P* (non-pink-eye), introduced into the 129/Sv genetic background, increased the incidence of teratomas in the 129/Sv-Sl^J sub-strain (to 7%), largely due to the influence of the mutant Steel gene (Stevens and Mackensen, 1961; Stevens, 1962). However, the 129/Sv-*CP* genotype used in these studies no longer has the Steel gene, but the males would still be expected to have a high relative incidence of spontaneous teratomas (around 1.0%: Stevens and Little, 1954). Although not attributable to the Steel gene, there may be

some other genetic basis explaining the permissiveness of primordial germ cells or early embryos to develop into malignant tumours and the capacity of embryos from the 129 strain to be isolated as immortal, undifferentiated ES cells in tissue-culture.

The C57BL/6 strain has been shown to be teratocarcinoma-nonpermissive, whereas CBA/J embryos yield a high proportion of teratocarcinomas (Solter *et al.*, 1979). The low yield of ES cell lines from F_2 embryos in this study may have been due to the fact that to produce the F_1 generation, C57BL/6 females were mated to CBA/Ca males. Evidence from F_1 crosses has suggested that embryos are only permissive to teratocarcinogenesis if the mother of the F_1 hybrid embryo is of a permissive strain (Damjanov and Solter, 1982). Thus, the F_2 (C57BL/6 X CBA/Ca) embryos might have been expected to produce teratocarcinomas at a low frequency and perhaps also, have limited stem cell potential *in vitro*. This could be further examined by culturing embryos from the reciprocal cross (*i.e.* CBA/Ca X C57BL/6) to capitalise on any maternal epigenetic effect, by utilising the more permissive CBA strain as the dams for the original F_1 stocks. Caution is required when extrapolating from teratocarcinomas to ES cells, as the embryos of the C57BL/6 mouse strain have been found to yield ES cell lines at a high efficiency (10%) compared to the 129/Sv strain (5%; Doetschman *et al.*, 1985).

It may be informative to culture embryos obtained from reciprocal 129/Sv-*CP* X F_1 (C57BL/6 X CBA/Ca) matings. If such a genetic comparison were to be conducted, however, it would be sensible to use inbred mouse strains with high and low potentials to give rise to ES cells for the reciprocal matings. Utilising the C57BL/6 X CBA/Ca crossbred animals would make any interpretation of the data ambiguous, because of the large genetic heterogeneity in the F_1 and F_2 generations. If embryos produced from such reciprocal matings are no longer non-permissive, in terms of ES cell isolation, it may indicate the presence of dominant alleles (of maternal or paternal significance) from the 129/Sv-*CP* genome which may have genetic (and/or epigenetic) influences on the capacity of the cells of the ICM to proliferate in an undifferentiated state *in vitro*.

Embryos that had undergone a period of implantational delay were originally utilised by Evans and Kaufman (1981) to isolate undifferentiated stem cells into culture. The culture of delayed embryos has been reported to yield a three-fold increase in the efficiency of ES cell isolation compared to non-delayed embryos cultured in that laboratory by different people (Robertson and Bradley, 1986). An increase of a similar magnitude with delayed embryos was found in this study. Evans and Kaufman (1981) recovered embryos four to six days following ovariectomy (*i.e.* 6.5 to 8.5 days *p.c.*), however, they did not specify any differences in ES cell isolation with regard to the length of the implantational delay. Here, it was found that a

significant increase was obtained only after a six day interval following ovariectomy. An important observation from the use of the F_2 (C57BL/6 X CBA/Ca) embryos in this study, was that the naturally low capacity of the ICMs from this genotype to proliferate in culture was largely overcome following a period of implantational delay.

It is possible that the increase in the potential of delayed embryos to yield ES cells may have arisen either as a consequence of an increase in the number of cells in the ICM, or as a result of some epigenetic change in gene expression.

It is not known whether ES cells originate from a sub-population of the ICM or if all of the cells of the ICM have the potential to proliferate *in vitro*. The chimaeric clonal analysis of day 4.5 *p.c.* primitive ectodermal cells suggests that the majority, if not all, these cells are equally pluripotent (Gardner *et al.*, 1985). Thus, it might be expected that all 20 cells of the day 3.5 *p.c.* ICM (Copp, 1982) have an equal chance of being maintained as ES cells. However, in view of the findings presented in chapter five, suggesting that an epigenetic modification is required for the establishment of ES cells, not all of the cells of the ICM may undergo the modification necessary to maintain the stem cell phenotype *in vitro*. The calculations performed by Suda and colleagues (1987) on the increase in the population of undifferentiated cells from the embryonic outgrowth to the first passage colonies, have suggested that the ES cells in the cultures were originally derived from at least four cells of the day 3.5 *p.c.* ICM. Observations made here have recorded very few instances where disaggregated ICMs have resulted in the majority of colonies in the first passage cultures possessing an ES-like morphology. Typically, only one or two colonies may possess the stem cell phenotype, the remainder having differentiated. Increasing the number of cells in the ICM component, may increase the likelihood of establishing a progressively growing ES cell culture. One test of whether the cell number of the ICM does have a significant effect on the isolation of ES cells, could be to aggregate several cleavage-stage embryos together to produce "giant blastocysts", with a larger ICM component.

In delayed blastocysts, studies have shown there to be a small, but significant, increase in the ICM number compared to non-delayed, day 3.5 *p.c.* blastocysts (Copp, 1982). Over the period of the implantational delay there was a slow, gradual increase in the number of cells in the ICM, up to a maximum of around 32 cells five days after ovariectomy (compared to around 21 cells on day 3.5 *p.c.*). The absolute cell number of the ICM declined thereafter, due to the decreasing mitotic rates and to the high cell death rates (Copp, 1982). However, in these experiments no mention was made of the presence of endodermal cells. Gardner and co-workers (1988) have shown that the delamination of primitive endoderm, from the ICM, had occurred in all delayed blastocysts examined and in this regard they were similar to the day 4.5 *p.c.* embryo

(Gardner and Rossant, 1979). It therefore seems unlikely that all of the cells counted in the ICM by Copp were pluripotent.

The effect of implantational delay on ES cell isolation may have been due to a change in the pattern of gene expression. Although primitive endoderm has formed in the implantationally delayed embryo, a parietal endodermal layer has not been observed and no further differentiation of the ICM occurs (Gardner *et al.*, 1988). Therefore, the normal pattern of expression of genes responsible for the differentiation of the ICM is halted, possibly by a uterine inhibitor of blastocyst activity (Aitken, 1977). And so, the embryos enter a period of diapause within the uterine lumen. When these delayed embryos are recovered and placed into suitable culture conditions, although the serum proteins in the culture medium may be sufficient to terminate the diapause (Aitken, 1977), perhaps the genes responsible for differentiation are not activated by transcriptional factors. This disruption in the normal developmental sequence of gene expression, may be an essential feature in the establishment of ES cell lines *in vitro*; and may explain the observations of the higher proportions of both first passage colonies and permanent cell lines of undifferentiated morphology, from cultures of delayed embryos compared to non-delayed day 3.5 *p.c.* embryos (figure 3.2). The hypothesis that an epigenetic modification may be a pre-requisite for the isolation of ES cells into culture is a subject that is discussed further in chapter five.

One ES cell line isolated from an F_2 delayed embryo was shown to be pluripotent *in vivo*, forming chimaeric offspring. The frequency of chimaerism from the $F_2$18 line (28%) was significantly lower ($P<0.05$) than that for **all** seven ES cell lines derived from day 3.5 *p.c.* blastocysts from the 129/Sv-*CP* mouse strain which were tested in chapter five (see Table 5.1). As only one F_2 cell line was examined here, it can not be generalised that the capacity to participate in normal embryonic development is lower with ES cells derived from the C57BL/6 X CBA/Ca genotype. The slight shift in the sex ratio in favour of males indicated probable instances of "sex conversion", where XY stem cells injected into XX embryos resulted in the development of phenotypic males (Robertson, 1986). In two instances the reproductive organs of both sexes developed and resulted in the formation of overt hermaphrodites. This presumably occurred as a consequence of insufficient XY stem cells migrating to the female germinal ridge, to completely transform the sex of the developing foetus.

There have been only two basic mouse strains cited in the literature from which germline ES cell-derived chimaeras have been produced; namely, 129 (including a number of sub-strains; *e.g.* 129/Sv//Ev: Bradley *et al.*, 1984) and CD-1 (Suda *et al.*, 1987). The limited breeding trials conducted in this study with chimaeras produced from one 40XY ES cell line, derived from an F_2 (C57BL/6 X CBA/Ca) embryo, failed

to demonstrate that the F_2 derived stem cells had colonised the germ cell lineage. However, there is no biological reason to assume that karyotypically normal ES cells derived from any mouse genotype would not have the potential to form functional gametes. The extent of colonisation of the germ cell lineage may depend upon the competitive interactions between the cells from the two different mouse genotypes used to produce the chimaera (McLaren, 1976; Schwartzberg *et al.*, 1989).

The results presented in this introductory chapter have shown the ES cell culture system utilised here, to be both efficient (comparable to other laboratories) and reliable. Although there were some variations in the efficiency of ES cell isolation between different batches of embryos cultured, overall, the results were consistent. Thus, this chapter provides a baseline for the comparison of the results in the subsequent experimental chapters on murine and ovine ES cells.

CHAPTER 4

ISOLATION OF EMBRYONIC STEM CELLS FROM POST-IMPLANTATION-STAGE MOUSE EMBRYOS

4.1 INTRODUCTION

Experiments have been carried out to determine whether ES cells can be isolated from post-implantation-stage mouse embryos. Although there have been no previous reports, a number of findings have suggested that it might be possible to establish stem cells from such advanced developmental stages. Firstly, pluripotent cells are present within the developing mouse embryo up to day 7.5 *p.c.* (see chapter 1.1) and malignant teratocarcinomas have been produced from ectopic transplants of these embryonic stages (see chapter 1.2.3). Furthermore, EC cell lines have been isolated into tissue-culture from teratocarcinomas derived from embryos up to day 7.5 *p.c.* (McBurney, 1976). And, biochemical analyses have suggested that cultured pluripotent stem cells may share the greatest homology to the day 5.5 *p.c.* primitive ectoderm of the post-implantation-stage embryo (Martin *et al.*, 1978; Evans *et al.*, 1979; Lovell-Badge and Evans, 1980; Evans and Kaufman, 1983).

Commonly, ES cells have been isolated from the ICM of either day 3.5 *p.c.*, or implantationally delayed, blastocyst-stage mouse embryos. The only other embryonic stage from which murine ES cells have been isolated, has been from the individual blastomeres of decompacted morulae (Eistetter, 1989).

4.2 EXPERIMENTAL METHODS

Primitive ectoderm from day 5.5 *p.c.* post-implantation-stage embryos was isolated from superovulated six to eight week old virgin 129/Sv-*CP* females mated to 129/Sv-*CP* stud males, utilising procedures similar to those described by Hogan, Costantini and Lacy (1986a).

Females were sacrificed by cervical dislocation and the belly region swabbed with 70% alcohol. The abdominal cavity was then opened to reveal the reproductive tract. In turn, each uterine horn was cut just below the uterotubal junction. Grasping the end firmly with forceps, the uterus was pulled taut and scissors used to tear away

the mesometrium. Next, the uterine horn was opened by sliding the tip of a pair of iris scissors down along the anti-mesometrial wall of the uterus, moving towards the cervix. Keeping the uterus pulled taunt, forceps were used to "shell" the decidua (containing the embryos) out of the uterus. The decidua were placed into a dish containing DMEM with 10% bovine serum, antibiotics (50 IU/ml penicillin and 50 µg/ml streptomycin; Sigma) and 20mM HEPES buffer, added to maintain the pH during subsequent manipulations outside the incubator.

Under a dissecting microscope, each deciduum was teased apart with flame sterilised watchmakers forceps, to dissect out the early egg cylinder-stage embryo; easily identified as a dark speck from the trophoblast tissue. A representative illustration of the dissected embryo is shown in figure 4.1. The trophoblast and parietal endoderm tissues were torn away from the implantation site during the dissection procedure and hence, appeared ragged. A pro-amniotic cavity had often developed by this stage and a clear division between the primitive ectoderm and the

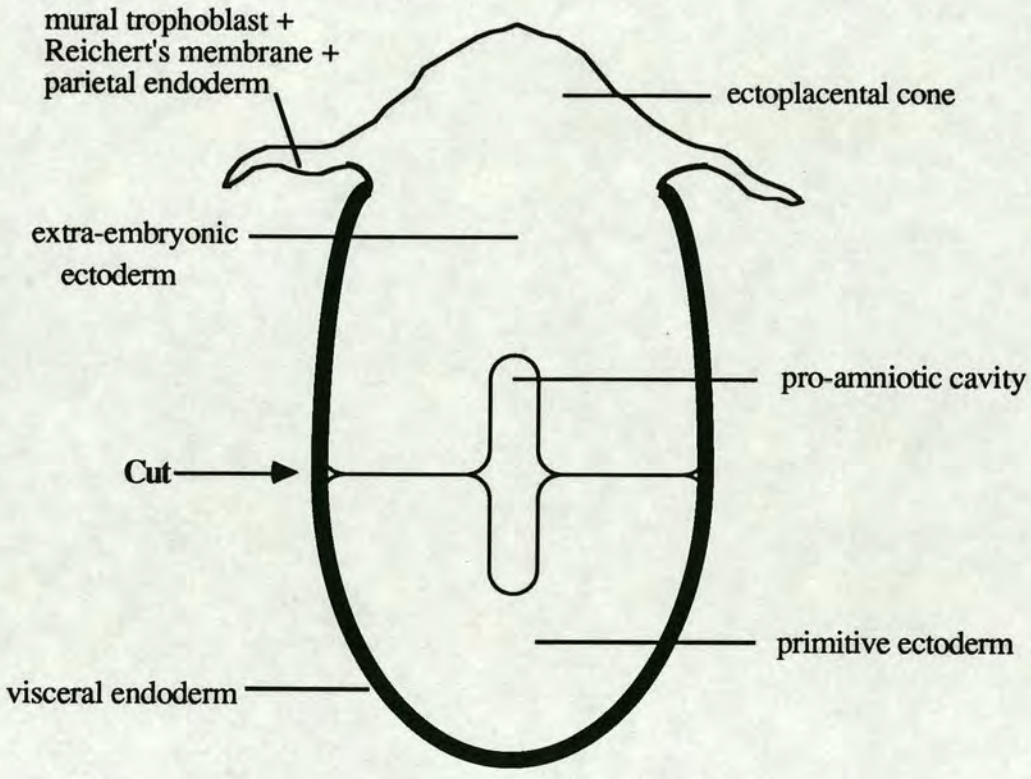


Figure 4.1: Representative illustration of a day 5.5 *p.c.* egg cylinder-stage mouse embryo following dissection from the deciduum, showing the position of the lateral cut made in order to isolate the embryonic portion of the embryo. (Adapted from: Rossant, Gardner and Alexandre, 1978).

extra-embryonic ectoderm (of trophectodermal origin) was often observed.

Isolation of pure primitive ectoderm tissue was accomplished by firstly, cutting away the the extra-embryonic regions of the egg cylinder utilising a fine glass microneedle constructed from a hand-pulled, Pasteur pipette ("cut" in figure 4.1). Then, the overlying layer of visceral endoderm was removed with an enzymatic pre-treatment. After rinsing in phosphate buffered saline (PBS), the tissue was placed in Ca^{2+} and Mg^{2+} free PBS containing 2.5% (w/v) pancreatin (porcine; Sigma), 0.5% (w/v) trypsin (porcine 1:250; Sigma) and 0.5% (w/v) polyvinylpyrolidone (M_r 10 000), further supplemented with 0.1% (w/v) D-glucose, 1% of a 100X stock solution of non-essential amino acids (Flow Laboratories) and 20mM HEPES buffer (pH 7.4: Hogan *et al.*, 1986b). The tissue was incubated for five minutes in a drop of this pancreatin-trypsin solution at 4°C. The dish was then flooded with DMEM containing 10% bovine serum to neutralise the crude proteases and the tissue transferred to a fresh droplet of ES_{20} medium (see chapter two, Table 2.1) under paraffin oil and incubated for one hour at 37°C. Following this recovery period, the endoderm layer was cleanly separated from the primitive ectoderm by gently aspirating the tissue up and down inside a fine, flame-polished Pasteur pipette. This treatment yielded relatively pure clumps of ectoderm which were then either explanted intact into tissue-culture for ES cell isolation, or were firstly disaggregated into small pieces, comprising several cells each, by incubating the ectoderm in TEG (chapter two, Table 2.2) for three to five minutes at 37°C followed by mechanical dissociation, using a fine pipette.

Apart from the timing of the first passage, methods for the establishment and maintenance of primitive ectoderm-derived ES cells were identical to those described previously (see chapters 2.3 and 2.4).

4.3 RESULTS

Two permanent ES cell lines were established from a total of 79 day 5.5 *p.c.* primitive ectoderm cultures. These experiments were conducted at the same time as those reported in chapter three, utilising the same batches of media and STO feeder cell layers. Thus, comparing the respective efficiencies of ES cell isolation for the 129/Sv-*CP* mouse strain, primitive ectoderm (2.5%) was a significantly ($P<0.025$) less efficient embryonic stage of development than day 3.5 *p.c.* embryos (13.2%).

Primitive ectoderms, from individual embryos, were explanted intact in 47 of the 79 original cultures whereas in the remaining 32, the ectoderm was firstly trypsinised and disaggregated mechanically into smaller cellular pieces. One ES cell line was obtained from each method. The isolation history for both of these lines was similar and is illustrated in figure 4.2 for the ECTO 3a line, isolated from intact primitive

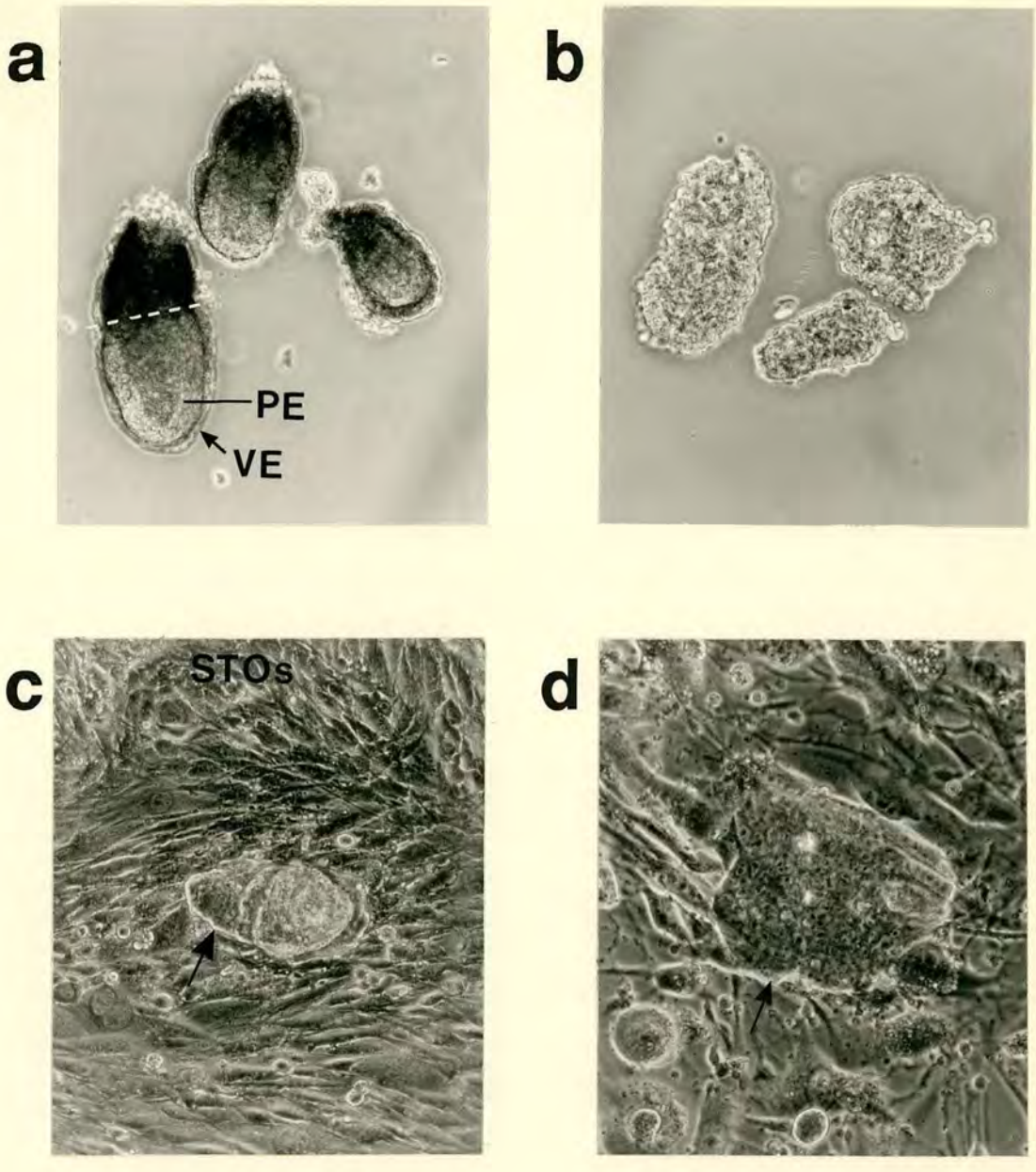


Figure 4.2: Establishment of the ECTO 3a ES cell line from day 5.5 *p.c.* primitive ectoderm. Egg cylinder-stage embryos were dissected from the decidua on day 5.5 *p.c.* (a). Primitive ectoderm (PE) tissue (b) was isolated from the embryos by firstly cutting away the extra-embryonic region along the dotted line in (a) and then utilising an enzymatic procedure to remove the overlying visceral endoderm (VE). Ectoderm was then explanted onto the STO feeder cells (c). From the undifferentiated outgrowth (arrowed) in (c), colonies of ES cell morphology were observed at the second passage (d) and subsequently expanded into a permanent stem cell line. (Magnification: x100)

ectoderm. The day 5.5 *p.c.* egg cylinder-stage embryo in figure 4.2a shows the position of the cut made to remove the extra-embryonic region and location of the visceral endoderm tissue overlying the primitive ectoderm. The primitive ectoderm was isolated as described in section 4.2 and resulted in relatively pure clumps of tissue (figure 4.2b) which were then explanted onto STO feeder layers prepared in microdrops. After two or three days of culture, undifferentiated outgrowths were commonly observed (as with ECTO 3a: figure 4.2c) which were then lightly disaggregated with TEG, before any obvious signs of differentiation, into several small pieces each comprising around 10-15 cells. Despite this early first passage, extensive differentiation often resulted in the cultures, with many endodermal and flattened epithelial-like colonies developing; however, trophoblast colonies were rarely observed. After a six to seven day culture interval, first passage colonies which had remained undifferentiated were trypsinised into single-cells and passaged. From these cells, second passage colonies of typical ES cell morphology grew in the ECTO 3a culture (figure 4.2d). In the course of cell expansion, the ECTO 3a line became infected with bacteria, but was recovered by sub-cloning, producing the line designated ECTO 3a.1.

In suspension culture, the primitive ectoderm-derived ES cells differentiated into complex embryoid bodies. Based on cell morphology, these embryoid bodies comprised an inner layer of cuboidal ectoderm-like cells separated from an outer layer of presumed parietal endoderm, by a Reichert's membrane. With prolonged culture, these embryoid bodies became cystic.

In the ECTO 3a.1 cell line, 69% (25/36) of the cells examined at passage 15 were classified as having an apparently normal 40XX chromosome constitution (figure 4.3); with the remaining cells being pseudodiploid. The other primitive ectoderm-derived ES cell line was also female, however, by the 11th passage 85% (16/20) of the cells examined appeared to have lost one X chromosome (*i.e.* XX/XO).

To test whether these primitive ectoderm-derived cells, which appeared morphologically identical to ICM-derived ES cells, were truly pluripotent *in vivo*, they were re-introduced into early mouse embryos and assessed for chimaerism. Cells of the ECTO 3a.1 ES cell line, between passages eight and 10, were injected into MF1 strain, host blastocysts. The data are presented in table 4.1. Based on skin and coat pigmentation, 75% (12/16) of the pups were judged to be chimaeric. The contribution of ES cells to the skin was variable, ranging between 10-75%. A typical primitive ectoderm-derived ES cell (female) chimaera is shown in figure 4.4. Breeding from both male and female chimaeras to albino, MF1 mates has not demonstrated the transmission of the ES cell genotype through the germline (Table 4.2).

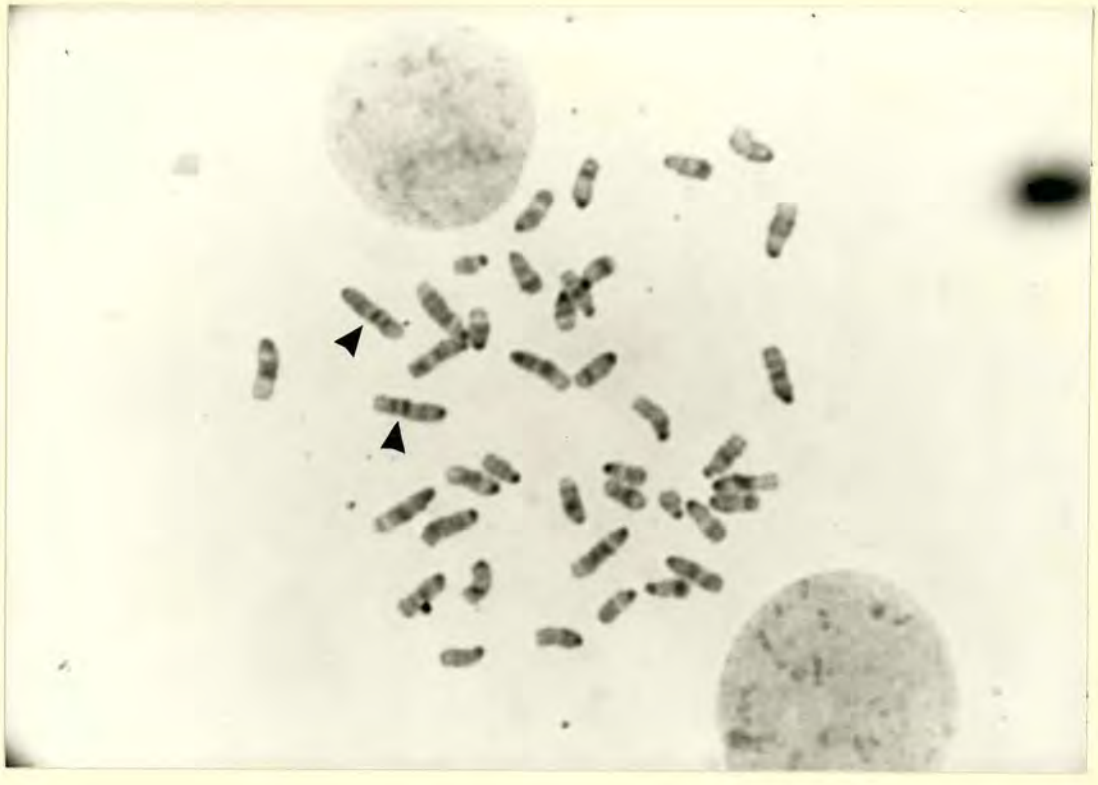


Figure 4.3: G-banded metaphase spread of the ECTO 3a.1 ES cell line at the 15th passage, showing an apparently normal 40XX chromosome constitution. The X chromosomes are indicated by the arrowheads. (Magnification: x1000)

Table 4.1: Production of chimaeras from a primitive ectoderm-derived ES cell line

ES Cell Line	Sex	Blastocysts Injected	Pups Born (%)	Chimaeras (%)	Sex Ratio male:female
Ecto 3a.1	XX	56	17[1] (30)	12 (75)[1]	8:4

1 Of these offspring, only 16 survived to an age to permit analysis for coat colour chimaerism.

Figure 4.4: Day 5.5 *p.c.* primitive ectoderm-derived embryonic stem cells are tripotent. The figure shows a female somatic chimaera produced following the injection of ES cells derived from the primitive ectoderm of a female day 5.5 *p.c.* 129/Sv-*CP* mouse embryo, into a day 3.5 *p.c.* albino, MF1 strain host blastocyst.

1 From the chimaera illustrated in figure 4.4, the primitive ectoderm-derived ES cells isolated from a 129/Sv-*CP* strain mouse embryo have contributed to three cell types based on pigmentation. Firstly, the melanocytes (derived from the neural crest) of the skin secrete eumelanin granules to produce the pigment in the hair. The agouti hair colour indicates an ES cell-derived mesodermal component in the hair follicles which transiently inhibits the production of the black pigment by the melanocytes, resulting in the sub-apical band of yellow. The third ES cell-derived component is observed from the pigmented melanocytes of the retina, formed from the neural ectoderm (Hogan *et al.*, 1986). To establish whether these primitive ectoderm-derived ES cells are pluripotent, a GPI isoenzyme analysis could be performed on various internal organs dissected from chimaeric mice.

Table 4.2: Breeding data from MF1-ECTO 3a.1 chimaeras backcrossed to the albino, MF1 mouse strain

	Litters	Offspring	Offspring		
			Albino	Black Agouti	Transmission (%)
Female Chimaera					
ECTO 3a.1 a	2	16	16	0	0
ECTO 3a.1 b	1	15	15	0	0
ECTO 3a.1 c	5	60	60	0	0
Totals	8	91	91	0	0
Male Chimaera					
ECTO 3a.1 p	6	71	71	0	0
ECTO 3a.1 q	8	73	73	0	0
Totals	14	144	144	0	0

4.4 DISCUSSION

This study has established that it is possible to isolate stable cell lines of ES cell morphology from day 5.5 *p.c.* primitive ectoderm, which are at least tripotent[1] and capable of forming somatic chimaeras. This is the first report of isolation of ES cells from post-implantation-stage mouse embryos.

The findings here and those of Eistetter (1989) indicate that there is a period of development, from day 2.5 to day 5.5 *p.c.*, from which pluripotent cells present within the murine embryo can be successfully isolated and maintained in culture. It may be possible to extend this upper limit and isolate ES cells from the primitive ectoderm of the day 7.5 *p.c.* embryo. A small number of primitive ectoderms (13) isolated from day 6.5 *p.c.* embryos were cultured here (data not presented), but only gave rise to well differentiated outgrowths and no progressively growing cultures; similar to earlier reports working with this stage and older day 7.5 *p.c.* embryos (Evans, 1981). Although day 5.5 *p.c.* primitive ectoderms often gave rise to outgrowths that appeared remarkably like established ES cells from the outset (as has been reported for immunosurgically isolated ICM outgrowths; Martin, 1981) most of these differentiated

extensively upon disaggregation. As the day 5.5 *p.c.* primitive ectoderm no longer has the potential to develop trophectoderm (Gardner, 1985) the rare instances were trophoblast colonies were observed, presumably arose from occasional contamination of cultures with extra-embryonic ectoderm, of trophectodermal origin (Rossant *et al.*, 1978). Because of the relatively large number of cells in the day 5.5 *p.c.* primitive ectoderm (approximately 120: Snow, 1977) and the problem of differentiation, some isolated ectoderms were lightly disaggregated prior to culture. However, this was no more successful in isolating cells of an undifferentiated morphology.

The findings of Gardner and colleagues (1985) have suggested that all of the cells of the day 4.5 *p.c.* primitive ectoderm may be equally pluripotent. However, it is not known whether this is true also for the day 5.5 *p.c.* primitive ectoderm and if all of the cells have the potential to give rise to ES cells.

The activity of the X chromosomes was not analysed in these studies. By day 5.5 *p.c.* it is generally accepted that in the female primitive ectoderm, inactivation of one X chromosome has been completed (Rastan, 1982). Many female ES cell lines isolated from the day 3.5 *p.c.* ICM have a partial deletion, or loss, of one X chromosome, thought to be a compensatory mechanism for the presence of two active X chromosomes (Robertson *et al.*, 1983a; Robertson and Bradley, 1986). The expectation that one X chromosome had been inactivated in the primitive ectoderms cultured here, might explain why one of the day 5.5 *p.c.* female ES cell lines possessed a high proportion of cells with a 40XX chromosome constitution. However, in the other female stem cell line the majority of the cells were XO.

The embryonic survival to term following the transfer of embryos injected with ES cells was low (30%) in these experiments. This was probably attributable to operator inexperience in embryo transfer, as the ECTO 3a.1 line was the first of the ES cell lines to be tested for *in vivo* pluripotency in this thesis. The proportion of pups which were overtly chimaeric, however, was relatively high (Evans *et al.*, 1985) and comparable to the frequencies obtained with ES cell lines derived from day 3.5 *p.c.*embryos of the 129/Sv-*CP* mouse strain tested in this thesis (see chapter five, Table 5.1).

As the ectoderm-derived ES cell lines are female, it has been difficult to detect germline transmission. The results from test breeding two male chimaeras suggests XX cells are incapable of producing functional spermatozoa (McLaren, 1976). The failure to detect germline transmission through the female chimaeras, could have

simply been due to the fact that only one ES cell line was examined and from the low numbers of females tested (3) and the number of progeny born (91); rather than any actual restriction in the developmental potential of primitive ectoderm-derived ES cells. It has been demonstrated that female ES cells, derived from a day 3.5 *p.c.* 129/Sv-*CP* strain embryo, are capable of entering the female germline when injected into MF1 host blastocysts (see chapter five, Table 5.2). It is unlikely that the germ cell lineage has differentiated from the primitive ectoderm by day 5.5. *p.c.* (Ginsburg *et al.*, 1990; and see chapter 1.4.1). And hence, it is also unlikely that the lack of germline transmission here was due to the chance that the ES cell line was derived from a theoretical sub-population of the primitive ectoderm no longer possessing the capacity to contribute to the gametes.

It is well documented that ES cells can contribute to all tissues in the chimaeric animal, including the gametes (Evans *et al.*, 1985). Examination of the full developmental potential of ICM-derived ES cells (Beddington and Robertson, 1989; Suemori *et al.*, 1990) has revealed that ES cells are also capable of colonising the trophectodermal and primitive endodermal lineages of the extra-embryonic membranes. These are tissues which mature ICMs are incapable of colonising (Gardner, 1985). Hence, it appears as though there may be some de-differentiation of ICM-derived ES cells *in vitro*. It would be informative to compare the capacity of ES cells derived from both earlier and later embryonic stages to colonise extra-embryonic tissues in chimaeric foetuses. This could determine whether the *in vivo* potential of the cell lines, derived from the three sources (individual blastomeres, ICM and primitive ectoderm), are "frozen" at their respective developmental stage of origin, or if they have converged in culture to the same cell lineage. The expectation, from the limited studies with EC cells, might be that the cell lines would be of differing pluripotency (see chapter 1.4.1). Thus, the primitive ectoderm-derived ES cell lines would not be expected to colonise the primitive endodermal and trophectodermal lineages (Gardner, 1985), if the cells are a true representation of the the developmental stage from which they are derived. However, from the *in vitro* differentiation data, these ectoderm-derived ES cell lines have the capacity to differentiate into embryoid bodies, apparently with an outer layer of parietal endoderm, presumed from the presence of an underlying layer of Reichert's membrane. This suggests that like ICM-derived ES cell lines, a de-differentiation event may also have occurred during the isolation of primitive ectoderm ES cell lines (Beddington and Robertson, 1989). If this is indeed the case, then there must have been some change in the pattern of gene expression during isolation. Perhaps this may be necessary for the isolation and

stable maintenance of ES cells in culture. Because the ectoderm-derived cell lines may not be representative of the day 5.5 *p.c.* primitive ectoderm within the embryo, this may limit any applications for the study of the molecular events of cellular differentiation at this stage of development. Nevertheless, the possibility of changes in the pattern of gene expression during ES cell isolation is intriguing and is a theme that is expanded in chapter five.

CHAPTER 5

THE EFFECT OF TREATMENTS EXPECTED TO PERTURB GENE EXPRESSION ON THE EFFICIENCY OF MURINE EMBRYONIC STEM CELL ISOLATION

5.1 INTRODUCTION

Broadly, there are two interpretations proposing how pluripotent cells can be diverted initially from their normal fate of differentiation within the early mouse embryo and maintained instead, as stable ES cell lines *in vitro* (discussed by Martin, 1980; Robertson and Bradley, 1986; see chapter 1.4). Either, ES cells may represent a normal embryonic lineage that is naturally programmed to continue dividing until appropriate developmental signals induce the cells to differentiate; and in the cellular disorganisation resulting from the outgrowth of embryos in culture, the cells may "escape" from these regulatory signals and continue to proliferate. Alternatively, some form of reversible, epigenetic modification may be required for the isolation of ES cells into culture. In this chapter, treatments expected to perturb the normal pattern of gene expression have been utilised to test this latter hypothesis indirectly. The specific treatments that were used in this study include the exposure of embryos to hyperthermia, puromycin or 5-azacytidine.

5.1.1 The Heat Shock Proteins

Following exposure to temperatures a few degrees above the physiological optimum, the cells of all organisms exhibit similar massive changes in the patterns of transcription and translation; whereby they preferentially synthesise a specific set of evolutionary conserved polypeptides: the heat shock proteins (HSPs: Schlesinger, Aliperti and Kelley, 1982; Pelham, 1985; Lindquist, 1986). The pattern of induced protein synthesis varies between species, however, virtually every species examined has HSPs in the 60-70 and 80-90 kiloDalton (kD) range (Schlesinger *et al.*, 1982; Pelham, 1985). These proteins shall be termed HSPs in this chapter although they should strictly be termed general "stress proteins", as treatments other than heat

shock produce a similar cellular response; however, not all of the HSPs may be released in response to these different agents (Welch, 1990). Other inducers of the HSPs include agents which, like heat, directly damage proteins, such as heavy-metal ions, arsenite, amino acid analogues incorporated into peptides (Pelham, 1985) and the antibiotic puromycin (Hightower, 1980). Other stresses such as ethanol, glucose starvation followed by re-feeding the cells and hypoxia, are thought to perturb the intracellular environment and cause similar protein damage indirectly (Pelham, 1985).

Unstressed eight-cell, blastocyst and day 7.5 *p.c.* primitive ectoderm mouse embryonic cells all synthesise high levels of 89kD and 70kD proteins belonging to the heat shock family (Bensaude and Morange, 1983; Morange *et al.*, 1984). This spontaneous synthesis occurs via the constitutive expression of heat shock cognate genes which produce mRNAs distinct, but closely related, to those from the heat shock-inducible genes accounting for HSP synthesis during stress. In the mouse blastocyst, there are two major proteins of the "70kD HSP family". One (the 68kD protein) is not constitutively expressed at this stage but is strongly heat-inducible, while the other (the 70kD protein) is spontaneously synthesised and is only slightly heat-inducible (Morange *et al.*, 1984). There are also two distinct but related 89kD HSPs produced in the mouse blastocyst from differential gene expression. Both are synthesised in unstressed cells, however, only one is strongly induced by heat shock (Barnier *et al.*, 1987).

The function of the heat-induced HSPs appears to be in the protection and repair of the cellular damage (especially of ribosomes) caused by the otherwise lethal effects of heat. The 70kD HSP family of proteins are thought to aid the reassembly of thermally damaged ribonuclear proteins within the nucleolus (Pelham, 1985; Welch and Suhan, 1986). During recovery from the stress, the 70kD HSP proteins leave the nucleolus and are localised instead on the cytoplasmic ribosomes, where they may play some role in repairing the translational apparatus (Welch and Suhan, 1986). The 90kD HSP family have been postulated to bind to denatured proteins in the cytoplasm, preventing them from forming insoluble and potentially harmful precipitates (Pelham, 1985).

The molecular mechanisms regulating the heat shock response have been studied most extensively in the fruit fly *Drosophila* and it is with this genus that particular reference is now made. As there is strong evidence to suggest that the mechanism of activation of the heat shock genes is highly conserved between species, it is likely that similar mechanisms exist in mammalian cells (reviewed by Pelham, 1985). The heat shock genes may begin to rapidly transcribe mRNAs for the new set of proteins within four minutes of cells experiencing stressful conditions from exposure to elevated temperatures (Lindquist, 1986). The activation of the heat shock

genes appears to be mediated by the binding and phosphorylation of a heat shock transcription factor (HSTF) to a heat shock element (HSE) in the promotor of the heat shock gene (Pelham, 1985; Sorger, 1991). In *Drosophila*, it has been proposed that the activity of a pre-existing pool of HSTF is repressed in unstressed cells, by binding to existing HSPs (Sorger, 1991). During heat shock, the HSPs competitively bind to the denatured proteins, produced in high levels from the stress, which results in the dissociation of the HSTF-HSP complex. This in turn allows the HSTF to bind to the HSE, promoting increased HSP production (Sorger, 1991). During the period of heat stress, the cellular machinery is directed to producing the HSPs as rapidly as possible. As the heat shock genes are activated, transcription of most previously active genes is repressed (Spradling, Pardue and Penman, 1977). Pre-existing RNA messages are (reversibly) inactivated and sequestered from translation during heat shock, while the HSP mRNAs are translated very efficiently (Storti *et al.*, 1980). Once the cells are returned to their normal growth temperature and the HSPs have aided the repair of the cellular, thermally-induced damage, the excess HSPs may then bind to the HSTFs and therefore shut down the activity of the heat shock genes (Sorger, 1991). The time taken for general transcription and translation to return to what may the pre-heat shock pattern, depends upon the degree of severity of the preceding heat shock (Lindquist, 1986).

The high constitutive expression of the 70kD and 89kD heat shock cognate genes in early mouse embryos and EC cells (Bensaude and Morange, 1983; Morange *et al.*, 1984) and the strongly conserved structure of these two major HSPs between different species (Schlesinger *et al.*, 1982) suggests that these HSPs may have some general role in regulating the cellular processes in the cells of all species. The constitutive expressive of some HSPs appears to be developmentally regulated. When murine EC cells from the F9 cell line were induced to differentiate *in vitro* to form parietal endoderm, the spontaneous synthesis of the 89-HSP markedly decreased (Barnier *et al.*, 1987). Another link between HSP expression and differentiation was suggested from the results where the steroid molting hormone ecdysterone, increased the synthesis of low molecular weight HSPs in embryonic *Drosophila* cells (Ireland and Berger, 1982). Following genome activation in the mouse, the 68kD HSP is not spontaneously synthesised in the conceptus until day 8.5 *p.c.* and only in the placenta (Morange *et al.*, 1984; Kothary *et al.*, 1987). Subsequently, the 68kD HSP is synthesised in the yolk sac on day 11.5 *p.c.* and not in murine embryonic tissues until on day 15.5 *p.c.* and only in the kidney of the adult (Kothary *et al.*, 1987). Thus, the pattern of constitutive expression of the 68kD HSP suggests it has a role in development.

5.1.2 DNA Methylation

DNA methylation is an epigenetic regulatory mechanism thought to play a part in cell determination and differentiation (Jones and Taylor, 1980). DNA in early embryos is highly methylated at many cytosine residues, to form 5-methylcytosine (reviewed by Razin and Riggs, 1980). Hypomethylation within certain G-C rich promoters is generally correlated with the activation of the affected genes (reviewed by Razin and Riggs, 1980; Doerfler, 1983) although this is not universal (Hsiao *et al.*, 1984).

The 5-methylcytosine analogue 5-azacytidine can be incorporated into DNA during replication. However, 5-azacytidine cannot be methylated since the compound is substituted with a nitrogen atom at the 5 position of the pyrimidine ring and thus, cannot accept a methyl group (Jones and Taylor, 1980). 5-azacytidine also directly inhibits methyl-transferase activity; where only a 0.3% substitution of 5-azacytidine for cytosine in DNA may reduce the DNA methyl-transferase level by more than 95% (Creusot, Acs and Christman, 1982).

Treatment of tissue-cultured cells with 5-azacytidine during DNA synthesis, therefore, causes random hypomethylation of DNA and has resulted in clonally heritable changes in the activity of genes and differentiative programmes (reviewed by Doerfler, 1983). For example, the mouse embryonic fibroblast cell line C3H10T1/2 has differentiated into chondrocytes, adipocytes and skeletal muscle following exposure to this cytosine analogue (Konieczny and Emerson, 1984). 5-azacytidine has also reactivated suppressed genes present on an inactive human X chromosome in a mouse-human somatic cell hybrid tissue-culture line (Mohandas, Sparkes and Shapiro, 1981). It has been reported that there is no evidence to suggest a mutational basis for the 5-azacytidine-induced biological effects in mammalian cells (Doerfler, 1983), however, the analogue has been shown to cause the neoplastic transformation of mouse fibroblast cells (Benedict *et al.*, 1977).

Experiments were carried out here to investigate the effect of treatments which are expected to perturb gene expression (heat shock, puromycin and 5-azacytidine) on the isolation of murine ES cells.

5.2 EXPERIMENTAL METHODS

The general procedures utilised in this chapter for the isolation and maintenance of ES cells from blastocyst-stage mouse embryos were the same as those described previously (chapter 2.3 and 2.4). Embryos were recovered 3.5 days *p.c.* and both

expanded and hatched blastocysts were cultured in these experiments. The specific treatments that were utilised are detailed below.

5.2.1 Heat Shock Treatment

Day 3.5 *p.c.* blastocyst-stage embryos were recovered from the uteri of two mouse strains: from 129/Sv-*CP* females mated to 129/Sv-*CP* males and from F_1 (C57BL/6 X CBA/Ca) females mated to F_1 (C57BL/6 X CBA/Ca) males (*i.e.* F_2 embryos). Hyperthermia was achieved by transferring embryos into a 0.5ml Eppendorf tube containing 0.25ml of pre-heated ES_{20} medium (see chapter two, Table 2.1) in a waterbath (model SE15: Grant Instruments, Cambridge, U.K.) set at the experimental temperature. This treatment was conducted immediately after embryo recovery, but before the embryos were placed into tissue-culture. 129/Sv-*CP* strain embryos were heat shocked for 10 minutes at either 40°C, 42°C or 44°C, whereas F_2 (C57BL/6 X CBA/Ca) embryos were heat shocked for 10 minutes at 42°C. Control embryos were handled in the same fashion and incubated in the waterbath at 37°C, for 10 minutes. After treatment, the embryos were retrieved from the Eppendorf tubes and each embryo was randomly allocated, along with control (non-heat shocked) embryos, to a coded STO feeder microdrop for culture.

5.2.2 Glycerol Pre-Treatment

Prior to heat shock, day 3.5 *p.c.* 129/Sv-*CP* strain blastocysts were exposed at room temperature to ES_{20} medium (with 20mM HEPES buffer) containing glycerol (Sigma) in two increasing concentration steps of 0.5M and 1.0M glycerol, for 15 minutes each. Following hyperthermia at 42°C for 10 minutes, the glycerol was diluted out of the embryos at room temperature in four 10 minute steps, decreasing the glycerol concentration in the medium by 0.25M each time. Embryos from the three treatment groups; those that had been heat shocked (positive control), glycerol pre-treated prior to heat shock, or non-heat shocked (negative control) embryos, were randomly allocated to individually coded microdrops for culture.

5.2.3 Puromycin Treatment

Day 3.5 *p.c.* embryos from the 129/Sv-*CP* strain were pre-incubated for 45 minutes in ES_{20} medium containing 0, 25, 75 or 150 μg/ml of puromycin dihydrochloride (Sigma) at 37°C. A 2.5mg/ml stock solution of puromycin was prepared in phosphate buffered saline, stored at 4°C and replaced after three weeks.

Media, containing the experimental concentrations of puromycin, were prepared immediately prior to use. After incubation, the embryos were then rinsed twice in fresh ES_{20} medium before being explanted into tissue-culture. In this experiment, the embryos from the four treatments were not randomly allocated to the STO microdrops.

5.2.4 5-Azacytidine Treatment

Day 3.5 *p.c.* 129/Sv-*CP* strain embryos were incubated for 24 hours in ES_{20} medium containing a range, from 10, 1, 0.5, 0.1, 0.05, 0.01 to 0.001 μM concentrations of 5-azacytidine (Sigma) to initially test the toxicity of this drug. A 1mM stock solution of 5-azacytidine was prepared in phosphate buffered saline and stored at 4°C for three weeks before it was replaced. Media, containing the required final concentration of 5-azacytidine, were prepared immediately before use. Following incubation, the embryos were then rinsed twice in fresh ES_{20} medium before being randomly allocated to coded STO feeder microdrops. Subsequent experiments, with both 129/Sv-*CP* and F_2 (C57BL/6 X CBA/Ca) embryos, were conducted with medium containing 0.1 or 0.05μM 5-azacytidine.

5.3 RESULTS

5.3.1 The Effect of Heat Shock on the Efficiency of ES Cell Isolation

The efficiency of ES cell isolation was increased by subjecting day 3.5 *p.c.* embryos, from the 129/Sv-*CP* mouse strain, to a short period of heat shock, immediately prior to culture (figure 5.1). When the embryos were exposed to an increase in temperature from 37°C to 42°C for 10 minutes, the yield of ES cell lines was significantly ($P<0.025$) increased compared to control embryos (13/48=27.1 ± 0.9% *vs* 7/79=8.9 ± 1.5%; n=3). No significant increase in the frequency of ES cell isolation was observed following a 10 minute exposure of embryos to 40°C (3/28=10.7%; this treatment was included in only one replicate of the experiment). The yield of ES cell lines resulting from a heat shock at 44°C was highly variable amongst the three batches of embryos exposed to this treatment (11/69=15.9 ± 7.2%) and was not significantly different compared to control embryos (figure 5.1). This variability was due partially to the effect the treatment had in causing a high relative incidence of embryonic death within the zona pellucida. In addition, heat shock at 44°C frequently resulted in outgrowths without, or very slowly growing, ICMs. These losses accounted for 24% of embryos in one replicate of the experiment.

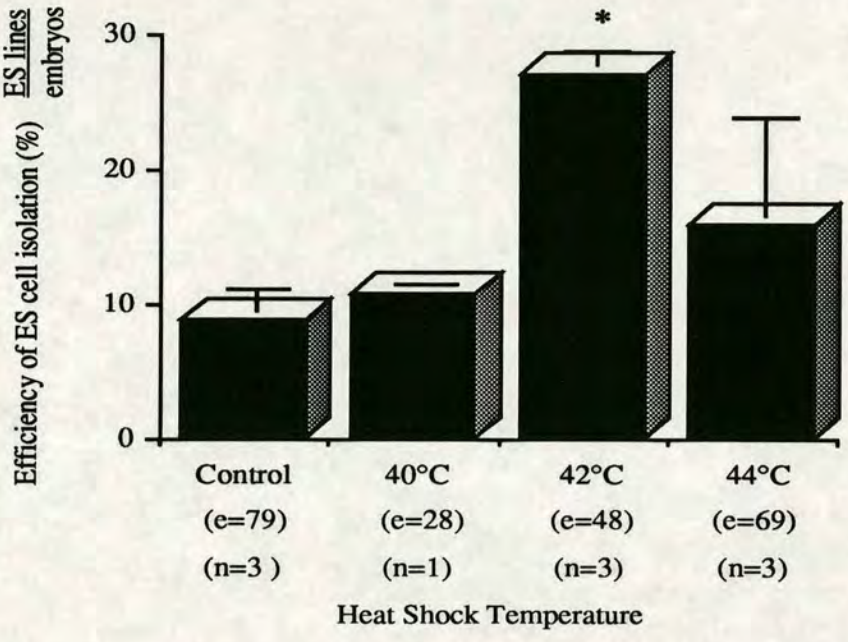


Figure 5.1: The effect of heat shock on the efficiency of ES cell isolation from day 3.5 *p.c.* embryos of the 129/Sv-*CP* mouse strain.
(mean ± s.e.m.; e = embryos; n = replicates) * P<0.025 compared to control

The survival profiles showing the proportion of control embryos and embryos exposed to a 42°C heat shock yielding ES-like colonies at each passage in culture, are shown in figure 5.2. The increase in ES cell isolation resulting from embryos heat shocked at 42°C did not become statistically significant until the third passage; where all but one heat shock-derived cell "line" was stably maintained thereafter. In the course of establishment of the 42°C heat shock-derived ES cell lines, there were small increases which accumulated over the early phases of isolation and ultimately resulted in the significant difference compared to controls (figure 5.2). The heat shocked embryos produced on average a slightly higher proportion of ICM outgrowths, which additionally resulted in more ES-like colonies at the first passage than from control embryos (24/48=50.0 ± 6.6% *vs* 30/79=38.0 ± 2.7%, respectively; P>0.05). Furthermore, these heat shock-derived ES cell primary colonies were of a significantly (P<0.05) more stable morphology *in vitro* than those derived from control embryos. Of the 30 control embryos giving rise to first passage cultures containing ES-like colonies, only colonies from seven embryos (or 23.3%) maintained a stable ES cell morphology over the following three passages and were expanded into permanent ES cell lines (figure 5.2). The first passage ES-like colonies from the

remaining 23 control embryos were lost to differentiation. With blastocysts that had been heat shocked at 42°C, however, permanent ES cell lines were derived from 13 of the 24 original heat shocked embryos which yielded ES-like colonies at the first passage (*i.e.* 54.2%).

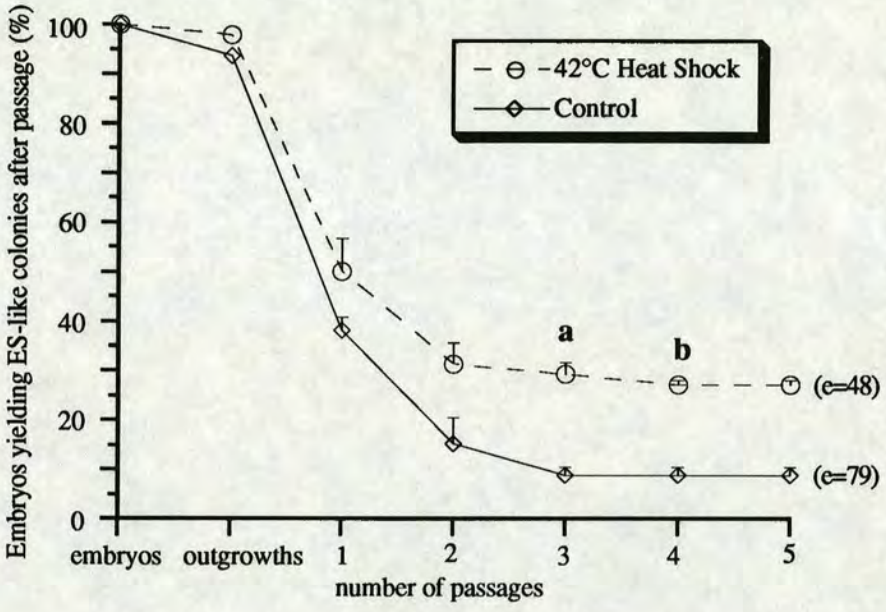


Figure 5.2: The survival profiles showing the percentage of control (37°C) and heat shocked (42°C) 129/Sv-*CP* strain mouse embryos giving rise to ES-like colonies after repeated passage in culture. After passage five, ES cells derived from both treatment groups were maintained as permanent ES cell lines.
(mean ± s.e.m.; e = embryos; n = 3) **a** $P<0.01$ and **b** $P<0.025$ compared to control

Because of the significant result obtained from applying heat shock to 129/Sv-*CP* strain embryos, the effect of elevating temperature on day 3.5 *p.c.* F_2 embryos, of the C57BL/6 X CBA/Ca genotype, was examined. No significant increase in the efficiency of ES cell isolation was observed between F_2 embryos that had been heat shocked at 42°C for 10 minutes (2/104=1.9%) and controls (0/107) in a single batch of embryos cultured. This result was not significant because of the low numbers of embryos cultured, given that the C57BL/6 X CBA/Ca genotype appears to be non-permissive in terms of ES cell isolation (chapter three). Both F_2 heat shock-derived ES cell lines were isolated from hatched blastocysts (2/39). Studies with 129/Sv-*CP* strain embryos heat shocked at 42°C following recovery on day 3.5 *p.c.*

have shown a small, although not significant, increase in the frequency of ES cell isolation from expanded (8/26=30.8 ± 3.0%) compared to hatched blastocysts (5/22=22.7 ± 1.8%; n=3). No significant embryo stage differences were observed with control 129/Sv-*CP* strain embryos from the same experiment (figure 5.1), where a similar proportion of expanded and hatched blastocysts yielded ES cell lines (5/60=8.3 ± 1.9% and 2/19=10.5 ± 4.6%, respectively; n=3).

Pre-treatment of day 3.5 *p.c.* embryos, from the 129/Sv-*CP* strain, with glycerol (acting as a thermo-protectant) prior to heat shock at 42°C, appeared to block the heat shock effect (figure 5.3). The yield of ES cell lines from embryos treated with glycerol + heat shock (2/28=7.1%) was no different compared to the negative control (2/20=10.0%), but was less than the heat shocked, positive control embryos (5/19=26.3%). The results from this single, small trial were not significant. However, as the proportions of embryos giving rise to ES cell lines in the positive and negative control groups were comparable to the levels described previously (see figure 5.1) the effect of the glycerol pre-treatment may have been real.

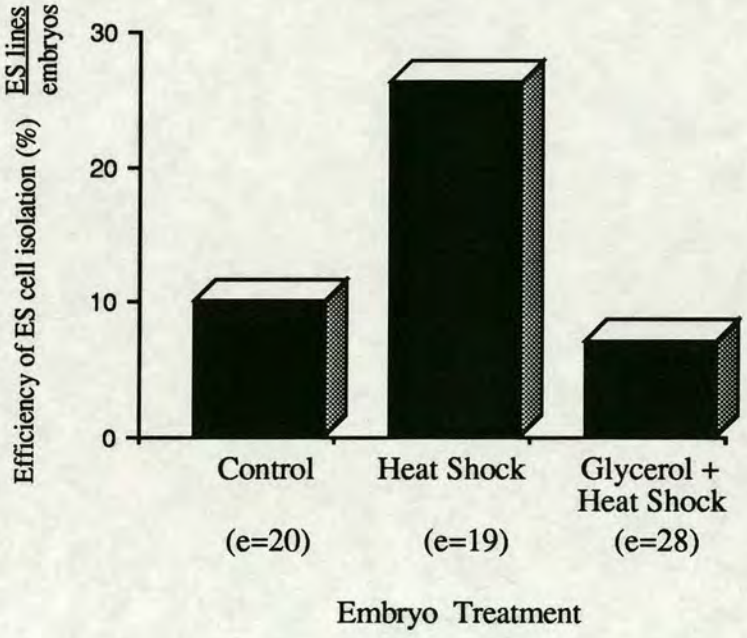


Figure 5.3: The effect of pre-treating day 3.5 *p.c.* 129/Sv-*CP* strain mouse blastocysts with glycerol prior to heat shock, at 42°C for 10 minutes, on the efficiency of ES cell isolation. (e = embryos; n = 1; P>0.05)

5.3.2 The Effect of Puromycin on the Efficiency of ES Cell Isolation

A 45 minute pre-incubation of day 3.5 *p.c.* embryos, from the 129/Sv-*CP* mouse strain, in medium containing the antibiotic puromycin significantly ($P<0.05$) increased the efficiency of ES cell isolation, at an optimal concentration of 25μg/ml (10/41=24.4%) compared to control embryos (5/61=8.2%; figure 5.4) in a single experiment. Treatment of embryos with higher concentrations of puromycin did not increase the proportion of embryos giving rise to ES cell lines above the yield obtained from the controls. A brief incubation of embryos in medium containing 75 or 150μg/ml of puromycin resulted in 11.6% (5/43) and 9.3% (4/43) of embryos yielding stem cell lines, respectively (figure 5.4).

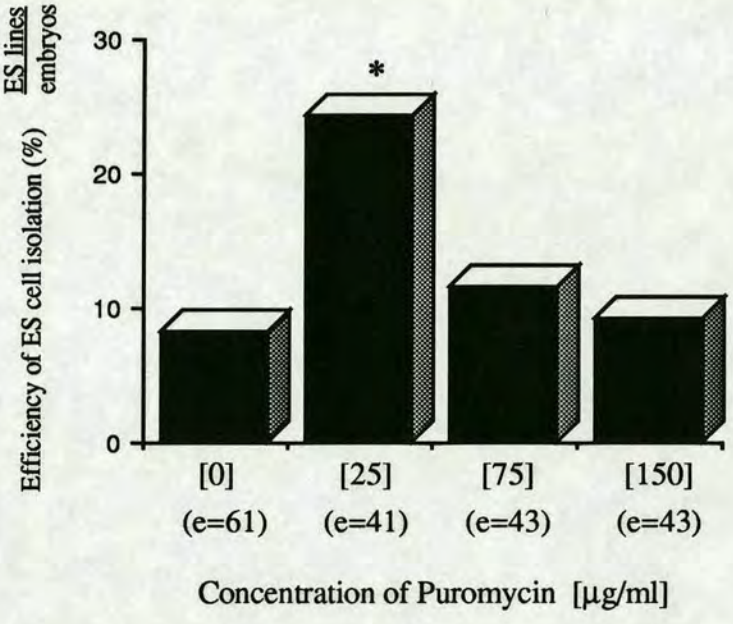


Figure 5.4: The effect of the pre-treatment of day 3.5 *p.c.* 129/Sv-*CP* strain mouse embryos with varying concentrations of puromycin on the efficiency of ES cell isolation. (e = embryos; n = 1) * $P<0.05$ compared to [0] control

The survival profiles of ES-like colonies derived from control embryos and those pre-incubated in 25μg/ml of puromycin are presented in figure 5.5. They both show a very similar pattern, however, a significantly ($P<0.025$) greater proportion of embryos gave rise to ES-like colonies at the first passage from the puromycin-treated group, compared to the control embryos (22/41=53.7% *vs*

18/61=29.5%, respectively). This was a difference which was maintained throughout the subsequent culture history (figure 5.5). There was no significant difference between the treatments with regard to the morphological instability of primary ES-like colonies in culture. Before the establishment of permanent ES cell lines at the third passage, a similar proportion of first passage ES-like colonies were lost to differentiation in both puromycin-treated and control embryo groups (12/22=54.6% *vs* 13/18=72.2%, respectively; P>0.05).

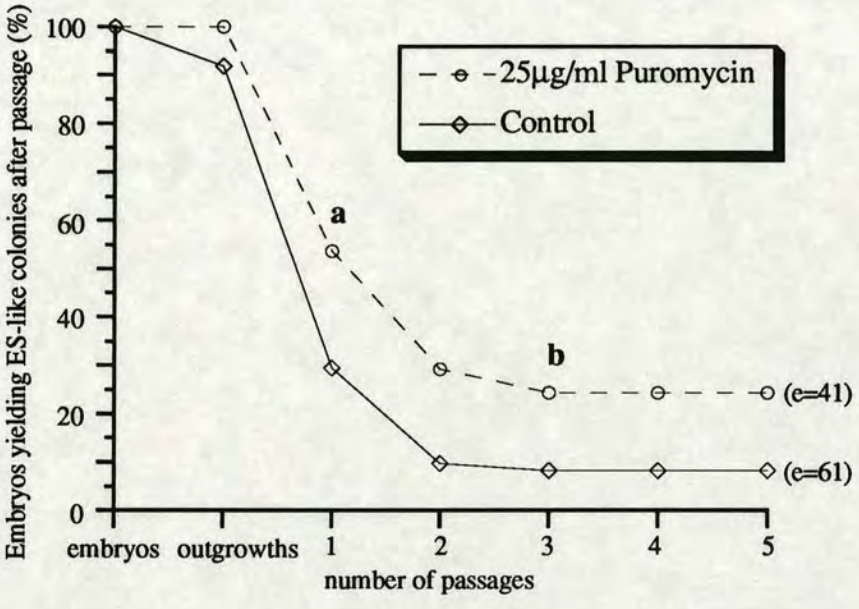


Figure 5.5: The survival profiles showing the percentage of control and puromycin [25µg/ml] treated 129/Sv-*CP* strain mouse embryos giving rise to ES-like colonies after repeated passage in culture. After passage five, ES cells derived from both treatment groups were maintained as permanent ES cell lines. (e = embryos; n = 1) **a** P<0.025 and **b** P<0.05 compared to control

5.3.3 The Effect of 5-Azacytidine on the Efficiency of ES Cell Isolation

Preliminary studies, where 129/Sv-*CP* strain embryos were incubated in medium containing one of seven different concentrations of 5-azacytidine, ranging from 0.001µM to 10µM (see section 5.2.4), indicated that the optimal concentration,

increasing the frequency of ES cell isolation, was between 0.05 and 0.1μM. Higher concentrations resulted in the expanded blastocysts collapsing within the zona pellucida and eventually dying, two or three days after the removal of the embryos from the 5-azacytidine medium. Lower concentrations of 5-azacytidine appeared ineffective in increasing the capacity of embryos to give rise to ES cell lines, compared to controls.

As there was no significant difference in the proportions of embryos yielding ES cell lines following incubation in either 0.05 or 0.1μM 5-azacytidine for 24 hours, the data from the two groups has been combined. From three batches of 129/Sv-*CP* strain blastocysts treated in this experiment, a twofold increase in the efficiency of ES cell isolation was observed with 5-azacytidine-treated embryos (13/57=22.8 ± 4.7%) compared to control embryos (5/49=10.2 ± 0.2%; P>0.05).

From the encouraging results presented above, day 3.5 *p.c.* F_2 (C57BL/6 X CBA/Ca) embryos were also treated with 0.1μM 5-azacytidine. This experiment was repeated twice and similar observations were made on each occasion. Although the blastocyst-stage embryos appeared to develop during the 24 hour incubation in the 5-azacytidine medium (becoming very expanded, with some embryos hatching from the zona), they failed to outgrow normally after being explanted onto the STO feeder cell layers. Many of the outgrowing embryos did not have an actively proliferating ICM and after four to six days of culture, two-thirds (32/51) of the outgrowths were composed entirely of flattened, giant trophoblast cells. The remaining outgrowths that were disaggregated did not produce any progressively growing ES-like cultures at the first passage.

5.3.4 Production and Analysis of Chimaeras

ES cells from lines derived from heat shocked, puromycin-treated or 5-azacytidine-treated embryos, plus one "control" (non-perturbed) stem cell line, were injected into the blastocoelic cavity of day 3.5 *p.c.* MF1 strain embryos, to examine their *in vivo* developmental potential. All stem cell lines studied here were isolated from 129/Sv-*CP* strain embryos and were injected between passages eight and 13. The results are summarised in Table 5.1 (refer to the legend for ES cell line nomenclature). All of the ES cell lines tested here produced chimaeric mice at a high frequency. An average of 75% of the pups, which survived to an age to permit assessment, were chimaeric. There were no significant differences between the seven independently derived lines in the proportion of offspring which were chimaeric, which varied from 63% to 92%. All but one of the cell lines showed a similar distribution in the contribution the (10 to 15) injected stem cells made to the coat of the

Table 5.1: Summary of the chimaera-forming efficiencies of murine embryonic stem cell lines derived from either heat shocked, puromycin-treated, 5-azacytidine-treated or control embryos

ES Cell Line	Chromosome Constitution	Blastocysts Injected	Pups Born (%)[1]	Chimaeras (%)[2]	Sex Ratio male:female
129 HS_{42}14	87% 40XX^{del}	44	18[3] (41)	11 (92)	6:5
129 HS_{42}20	86% 40XY	67	44[4] (66)	33 (77)	25:8
129 $P_{[25]}$4a	47% 40XX	15	11 (73)	9 (82)	2:7
129 $P_{[25]}$14a	95% 40XY	40	17[4] (43)	10 (63)	8:2
129 AC10	67% 40XY	44	20 (45)	13 (65)	10:3
129 AC19	89% 40XY	44	17[4] (39)	11 (69)	5:6
129 C1	88% 40XY	52	15[5] (29)	11 (92)	8:3
Totals		306	142 (46)	98 (75)	

1 includes embryos transferred to recipients that failed to become pregnant

2 excludes pups that were not analysed because of cannibalism

3 includes six pups that were cannibalised

4 includes one pup that was cannibalised

5 includes three pups that were cannibalised

ES cell line nomenclature:

129 embryos from the 129/Sv-*CP* strain

HS_{42} embryos heat shocked at 42°C

$P_{[25]}$ embryos treated in 25µg/ml puromycin

AC embryos treated in 0.1µM 5-azacytidine

C control (non-perturbed) embryo

chimaeras, varying between 10-90%. The skin of many of the animals comprised of an estimated 50% ES cell component. The one exception was the 129 AC19 ES cell line, where none of the chimaeras produced had a greater than 30% stem cell contribution to the coat.

Two of the ES cell lines injected into blastocysts were female: 129 $HS_{42}14$ and 129 $P_{[25]}4a$. Both female stem cell lines contained substantial populations of cells with chromosomal abnormalities, however, this did not preclude the ES cells from forming somatic chimaeras at a high efficiency (92% and 82%, respectively; Table 5.1). Approximately, 87% (13/15) of the cells of the 129 $HS_{42}14$ line (at passage nine) have a 75% deletion of one X chromosome distal to the region A3 (Nesbitt and Francke, 1973), with the remaining cells having completely loss the X chromosome. In the 129 $P_{[25]}4a$ line, approximately 47% (16/34) of the cells (at passage eight) have an apparently normal 40 XX chromosomal constitution (figure 5.6), with the remaining cells having either lost (39) or gained (41/42) chromosomes, in roughly equal proportions. The 129 $P_{[25]}4a$ line colonised the germline in one of the five female chimaeras that were test bred (Table 5.2), having produced one offspring with the black agouti coat colour, indicative of the 129/Sv-*CP* derived stem cells (figure 5.7).

The other five ES cell lines that were injected into blastocysts were male and contained high proportions of cells (between 67% and 95%; $n \geq 10$) which possessed an apparently normal 40 XY chromosomal constitution, between passages eight and 11 in culture (Table 5.1 and see figure 5.8). The remaining cell populations in each of these cell lines were pseudodiploid; no tetraploid cells were observed in the metaphase spreads. Generally, there was a shift towards phenotypic males in the chimaeric population following the injection of male ES cells, most noticeable with the 129 $HS_{42}20$ cell line (Table 5.1).

Male chimaeras derived from the XY ES cell lines 129 $HS_{42}20$, 129 AC10 and 129 C1 were mated to MF1 strain female mice to test for germline stem cell contributions. The results are presented in Tables 5.3, 5.4 and 5.5 for the three cell lines, respectively. Germline transmission was demonstrated in at least two chimaeras from these male ES cell lines, derived from either heat shocked, 5-azacytidine-treated or control embryos.

Two classes of germline male chimaeras were demonstrated. Two chimaeras (129 $HS_{42}20$**v** and 129 AC10**q**; see Tables 5.3 and 5.4, respectively) transmitted the dominant black agouti coat colour phenotype to all of their progeny (see figure 5.9). The remaining male germline chimaeras transmitted the 129/Sv-*CP* stem cell genotype in only a proportion of their sperm. Nine of the 22 phenotypic male chimaeras produced from the 129 $HS_{42}20$ ES cell line which were paired with mature

Figure 5.6: G-banded metaphase spread of the 129 $P_{[25]}$4a ES cell line at the eighth passage, showing an apparently normal 40XX chromosome constitution. The X chromosomes are indicated by the arrowheads. (Magnification: x1000)

Table 5.2: Breeding data from MF1-129 $P_{[25]}$4a female chimaeras backcrossed to albino, MF1 strain males

Female Chimaera	Litters	Offspring	Offspring: Albino	Offspring: Black Agouti	Transmission (%)
129 $P_{[25]}$4a a	2	19	19	0	0
129 $P_{[25]}$4a b	3	26	26	0	0
129 $P_{[25]}$4a c	3	17	16	1	5.9
129 $P_{[25]}$4a d	2	14	14	0	0
129 $P_{[25]}$4a e	2	22	22	0	0

Figure 5.7: Female germline chimaera and offspring. The chimaera was produced following the injection of ES cells at the eighth passage from the female 129 $P_{[25]}4a$ line into a host MF1 strain blastocyst. Transmission of the black agouti 129/Sv-*CP* stem cell-derived genotype, through the female germline, was demonstrated in one pup in a litter of six resulting from mating with an albino, MF1 strain male.

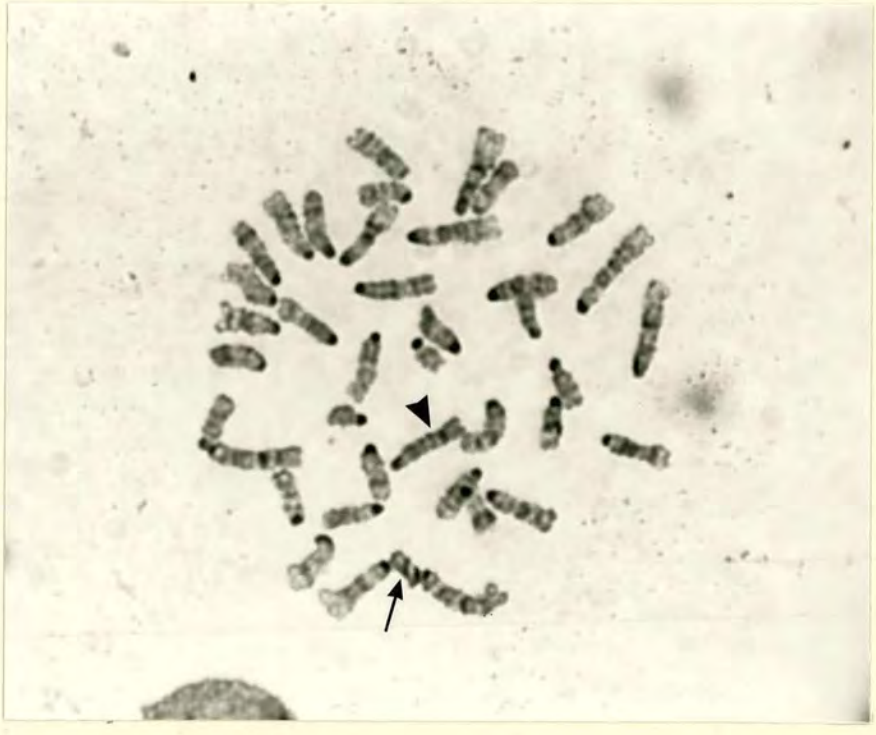


Figure 5.8: G-banded metaphase spread of the 129 $HS_{42}20$ ES cell line at the 11th passage, showing an apparently normal 40XY chromosome constitution. The X chromosome is indicated by the arrowhead and the Y chromosome by the arrow. (Magnification: x1000)

Table 5.3: Breeding data from MF1-129 $HS_{42}20$ male chimaeras backcrossed to albino, MF1 strain females

Male Chimaeras	Litters	Offspring	Offspring: Albino	Offspring: Black Agouti	Transmission (%)
129 $HS_{42}20$ p	Did not breed				
129 $HS_{42}20$ q	Did not breed				
129 $HS_{42}20$ r	2	19	19	0	0
129 $HS_{42}20$ s	Did not breed				
129 $HS_{42}20$ t	2	20	20	0	0
129 $HS_{42}20$ u	4	45	44	1	2.2
129 $HS_{42}20$ v	2	20	0	20	100
129 $HS_{42}20$ w	9	86	86	0	0
129 $HS_{42}20$ x	13	125	125	0	0
129 $HS_{42}20$ y	Did not breed				
129 $HS_{42}20$ z	Did not breed				
129 $HS_{42}20$ pp	2	25	24	1	4.0
129 $HS_{42}20$ qq	Did not breed				
129 $HS_{42}20$ rr	Did not breed				
129 $HS_{42}20$ ss	Did not breed				
129 $HS_{42}20$ tt	3	25	25	0	0
129 $HS_{42}20$ uu	1	7	7	0	0
129 $HS_{42}20$ vv	3	25	25	0	0
129 $HS_{42}20$ ww	1	3	1	2	66.7
129 $HS_{42}20$ xx	1	9	9	0	0
129 $HS_{42}20$ yy	Did not breed				
129 $HS_{42}20$ zz	3	34	34	0	0

Table 5.4: Breeding data from MF1-129 AC10 male chimaeras backcrossed to albino, MF1 strain females

Male Chimaeras	Litters	Offspring	Offspring: Albino	Offspring: Black Agouti	Transmission (%)
129 AC10 p	4	44	39	5	11.4
129 AC10 q	1	15	0	15	100
129 AC10 r	Did not breed				
129 AC10 s	1	14	14	0	0

Table 5.5: Breeding data from MF1-129 C1 male chimaeras backcrossed to albino, MF1 strain females

Male Chimaeras	Litters	Offspring	Offspring: Albino	Offspring: Black Agouti	Transmission (%)
129 C1 p	5	58	57	1	1.7
129 C1 q	4	41	41	0	0
129 C1 r	3	20	20	0	0
129 C1 s	4	44	44	0	0
129 C1 t	4	45	45	0	0
129 C1 u	2	21	19	2	9.5

Figure 5.9: Embryonic stem cells derived from embryos exposed to heat shock retain full pluripotent developmental potential. The figure shows a male germline chimaera transmitting the black agouti ES cell-derived genotype to all offspring, after mating with an albino MF1 strain female. The chimaera was produced following the injection of XY stem cells from the 129 $HS_{42}20$ ES cell line into a presumed XX host blastocyst, from the MF1 mouse strain. The male stem cells resulted in the "sex-conversion" of the mouse.

females, did not breed (Table 5.3). After autopsy, these nine chimaeras were found to have abnormal reproductive tracts, possessing both male and female sex organs. Of the fertile 129 $HS_{42}20$ male chimaeras, 31% (4/13) transmitted the stem cell genotype through their gametes.

5.4 DISCUSSION

The original hypothesis examined (indirectly) in this chapter was whether an epigenetic alteration is required for pluripotent cells from early mouse embryos to be established in culture as stable ES cell lines. Embryos were challenged with treatments, either hyperthermia, puromycin- or 5-azacytidine-containing medium, known to interfere with gene expression. The increased frequency of isolation of ES cells resulting especially from exposure to heat shock or puromycin, might suggest that the isolation of stem cells may depend upon reversible changes in the pattern of gene expression and the treatments utilised here may have helped to promote these changes in culture.

At present, the molecular mechanism(s) behind the action of these treatments are not understood. Although the results presented in this chapter have yet to be substantiated with protein synthesis and DNA methylation data, the following discussion shall assume that the heat shock and puromycin treatments did indeed result in the enhanced expression of the HSPs, as expected (Morange *et al.*, 1984; Lee and Dewey, 1987).

It is possible that by exposing embryos to a brief period of heat shock, the increase in the yield of ES cells occurred as a result of some change in the pattern of gene expression. Either genes necessary for the *in vitro* establishment of stem cells were activated or alternatively, genes responsible for the differentiation of the ICM were not transcribed and the normal sequence of gene activation in the embryo was interrupted by this treatment. By stressing the embryo with elevated temperature, the cells respond by transiently repressing general (37°C) protein synthesis (both transcription and translation) and instead activate the heat shock genes (Schlesinger *et al.*, 1982). The mRNAs are then rapidly and efficiently translated to produce the HSPs. In the mouse blastocyst, a 68kD HSP is induced by the short period of hyperthermia (Morange *et al.*, 1984) but whether differences exist between the trophectoderm and the ICM in the synthesis of the HSPs is not known. The broad function of these HSPs appears to be in protecting and repairing the ribosomes damaged from the stressful conditions (Pelham, 1985).

There is no evidence to suggest that the HSPs themselves are directly responsible for influencing the pattern of gene activation, once the temperature of the

cells has returned to 37°C and general protein synthesis has resumed. Some of the biochemical events associated with heat shock include a rapid decline in intracellular pH and a corresponding increase in cytosolic calcium levels (reviewed by Welch, 1990). These cellular changes may have roles in activating "second messengers" leading to the activation of gene transcription. Although changes in intracellular pH and calcium have been shown not to be required for activation of the heat shock genes (Welch, 1990) such cellular conditions may have the potential to modify the pattern of gene expression during the recovery of the cells.

In studies where rat foetuses (Higo *et al.*, 1989), human HeLa cells (Andrews *et al.*, 1987) and cardiac muscle (Izumo, Nadal-Ginard and Mahdavi, 1988) were stressed (by various agents) expression of the heat shock genes was accompanied by elevated expression of various proto-oncogenes. Proto-oncogenes are believed to have a role in controlling cell proliferative and differentiative processes (Bishop, 1983). One proto-oncogene (*c-myb*) has been shown to be expressed at high levels in EC cells and was rapidly inactivated following the induction of differentiation by retinoic acid (Lockett and Sleigh, 1987). It would be interesting to examine the pattern of proto-oncogene expression following the heat shock of blastocysts. The activation of certain proto-oncogenes may result in compensatory proliferation of the ICM, in response to the damage caused by the stress (as hypothesised for other cellular systems; Higo *et al.*, 1989). Although no actual measurements were taken of the size of the ICM outgrowth, there did not appear to be any obvious increase in this parameter after heat shock compared to control embryos.

As discussed above, the heat shock effect could have been due to enhancing the activation of certain genes (perhaps proto-oncogenes) responsible for maintaining the undifferentiated phenotype *in vitro*; or alternatively, the effect may have been due to genes responsible for differentiation not being transcribed, once the cellular genome was reactivated during recovery from the stress. This latter view could form part of the so-called "embryonic stress hypothesis of teratogenesis" with hyperthermia interfering with the normal sequence of gene expression during development (German, 1984). According to this hypothesis, early in teratogenesis there may be a failure of essential genes, required for subsequent differentiation, to be transcribed at some critical point in development because the cellular machinery has been diverted by the cellular stress into producing the HSPs instead. There are numerous reports of various developmental defects resulting from heat stressing mammalian foetuses *in utero*, dependent upon the developmental stage at which the stress was imposed (reviewed by Edwards, 1986). In most of these cases, hyperthermia acted as a teratogen by causing general cell death. However, there is some evidence to suggest that anatomical malformations may also be due to the induction of the HSPs, inhibiting

transcription of developmentally regulated genes in *Drosophila* (Mitchell and Lipps, 1978) and rat foetuses (Higo *et al.*, 1988). Perhaps exposing day 3.5 *p.c.* mouse embryos to heat shock at 42°C resulted in the production of the HSPs for a sufficiently long period to perturb the normal developmental sequence of transcription, preventing the activation of genes responsible for the differentiation of the ICM and so, increased the capacity of these cells to remain undifferentiated *in vitro*. This may be analogous to the view proposed as to why delayed blastocysts (where differentiation of the ICM has been arrested) result in higher frequencies of ES cell isolation than non-delayed embryos (see chapter three). The heat shock of embryos resulted in significantly greater morphological stability of first passage ES-like cell colonies during the isolation of stem cell lines; that is, they appeared to be less pre-disposed to differentiation compared to non-heat shocked control embryos (see figure 5.2). Such an effect may be very dependent upon the embryonic stage at which the stress is imposed. Following heat shock, there was a slightly lower (although not significant) yield of ES cell lines from hatched blastocysts (where some cells of the ICM may have already formed primitive endoderm; Nadijcka and Hillman, 1974) compared to expanded blastocysts.

Exposing blastocysts to heat shock may have enhanced the expression of the heat shock genes, which then indirectly disturbed the normal pattern of embryonic cell differentiation. In *Drosophila*, there is evidence to suggest a correlation between the degree of inhibition of differentiation in cultured embryonic cells and the level of induction of the HSPs in response to various stress agents (Bournias-Vardiabasis and Buzin, 1986). The production of the HSPs is positively correlated with the degree of stress induced (DiDomenico, Bugaisky and Lindquist, 1982). The three temperatures used in these studies may have resulted in different levels of HSP synthesis. The mild heat shock resulting from the 10 minute exposure of embryos to 40°C, may not have stressed the cells sufficiently to result in the production of the HSPs for the length of time required to block the transcriptional activation of genes causing the ICM to differentiate. Hyperthermia at 44°C, on the other hand, may have excessively prolonged the period of HSP production, killing the cells (Welch and Suhan, 1986). This may explain the observation that a substantial number of outgrowths from embryos heat shocked at 44°C did not contain an ICM component, as treatment at the high temperature may have caused cell death within the ICM. Heat shock at 42°C appeared to be optimal for inducing a sufficiently high heat shock response to perturb the normal programme of differentiation, but which was not deleterious to cell survival.

Pre-treatment of embryos with glycerol blocked the increase in the frequency of stem cell isolation resulting from heat shock (see figure 5.3). Glycerol has previously

been shown to block the release of the HSPs during hyperthermia in cultured chicken embryonic cells (Edington, Whelan and Hightower, 1989). Glycerol, as a polyhydroxyl alcohol, is thought to stabilise proteins by strengthening the hydrogen bonds of water molecules and hence, strengthening the hydrophobic interactions of proteins (Gekko and Koga, 1983). The chicken embryonic cells were therefore not stressed by the elevated temperature, as the glycerol protected heat sensitive targets from thermal damage. Thus, the transcriptional activation of the heat shock genes, both during and after the period of elevated temperature, were blocked (Edington *et al.*, 1989).

It is not clear whether the transcription and translation of normal (37°C) proteins would have been active during heat shock with glycerol-treated embryos. If general protein synthesis had been repressed during exposure to the elevated temperature and then reactivated once the embryos were returned to 37°C (without the release of the HSPs) this might imply that the heat shock effect, increasing ES cell isolation, may be due specifically to the HSPs themselves and not a general effect of disruption in the pattern of gene expression. If on the other hand, 37°C protein synthesis had continued during the heat shock of glycerol-treated embryos, it would not be obvious whether the phenomenon was due to the HSPs or a change in gene expression upon genome reactivation. An experiment which may clarify the point would be to treat the embryos, prior to culture, with a general, but reversible, inhibitor of transcription. A likely candidate would be the use of the antibiotic actinomycin-D, which is capable of penetrating intact cells, binding to DNA and preferentially blocking mRNA chain elongation (Lehninger, 1975a). DNA synthesis is not substantially affected by this drug (Lehninger, 1975a) and the actinomycin-D molecule may dissociate from the DNA upon replication and be diluted out of the embryonic cells. Therefore, actinomycin-D may be useful to block general transcription over the period when genes responsible for differentiation would normally becoming transcriptionally active in the ICM. Once the actinomycin-D is removed from the cells, the pattern of gene reactivation may not include those genes required for differentiation and consequently, this may confer some advantage towards the isolation of ES cells.

The brief incubation of mouse blastocysts from the 129/Sv-*CP* strain in medium containing 25µg/ml of the antibiotic puromycin resulted in a significant, three-fold increase in the efficiency of ES cell isolation compared to controls (see figure 5.4). This was similar to the effect of heat shock. The antibiotic puromycin is an analogue of aminoacyl-tRNA, blocking translation and resulting in the production of aberrant polypeptides. Puromycin interupts peptide chain elongation by its capacity to bind to the aminoacyl site on the ribosome when the peptidyl site is occupied with a tRNA (Lehninger, 1975b). A covalently bound peptidyl-puromycin derivative is formed

and dissociates from the ribosome, since it does not have the correct structure to move along to the peptidyl site to further elongate the peptide (Lehninger 1975b). The abnormal proteins formed from puromycin treatment have resulted in the induction of the HSPs in *Escherichia coli* (VanBogelen and Neidhardt, 1990), avian (Hightower, 1980) and mammalian cells (Lee and Dewey, 1987). Very high concentrations of puromycin (100μg/ml), similar to the highest treatments used in this study, are unlikely to result in the production of the HSPs because of the high frequency of peptide chain termination preventing their synthesis (Lee and Dewey, 1987). In accord with the classical heat shock response, general protein synthesis was reduced by up to 80% with treatment of Chinese hamster ovary cells with 30μg/ml puromycin (Lee and Dewey, 1987) and in the course of the response, general transcription would also be expected to have been transiently repressed, as a secondary effect. So, the increase in ES cell isolation resulting from puromycin-treated embryos (at a concentration of 25μg/ml) cannot be distinguished between an effect of the HSPs, or due to some change resulting from the general reactivation of the genome. However, since there was no increase in ES cell isolation from high concentrations of puromycin and as such treatment may not have caused the production of the HSPs but may have repressed transcription as a secondary effect, perhaps the increase in ES cell isolation observed at the lower concentration of puromycin was in some way due to the HSPs.

Although not significant, the incubation of blastocysts from the 129/Sv-*CP* mouse strain for 24 hours in medium containing 0.1μM 5-azacytidine, doubled the proportion of embryos yielding ES cell lines compared to control embryos. There appeared to be a difference between mouse strains in the cytotoxicity of this drug on pre-implantation embryos. In many instances, the treatment of embryos from the F_2 (C57BL/6 X CBA/Ca) genotype with 0.1μM 5-azacytidine resulted in cell death in the ICM, whereas detrimental effects of the drug on embryos from the 129/Sv-*CP* strain were only observed at between 10- to 100-fold higher concentrations. As a comparison of the DNA methylation status of control and 5-azacytidine-treated embryos, and ES cell lines, was not undertaken in these studies, it remains to be determined whether the increase in ES cell isolation from the 129/Sv-*CP* mouse strain was indeed due to hypomethylation and hence, gene activation. Exposure to 5-azacytidine has been shown to induce the HSPs in *Drosophila* embryonic cells (Bournias-Vardiabasis and Buzin, 1986). Substantially lower concentrations of 5-azacytidine (between 10- to 100-fold lower) were used in the studies here to allow for the survival of the ICM (from the 129/Sv-*CP* strain) compared to what previous workers have utilised in other *in vitro* systems to cause hypomethylation of DNA and gene activation (Mohandas *et al.*, 1981; Konieczny and Emerson, 1984). The lower concentrations of 5-azacytidine used here may not have caused hypomethylation, but

instead only stressed the cells sufficiently to induce the heat shock response. Thus, the mechanism responsible for the increase in ES cell isolation with 5-azacytidine may have been via a similar (but unknown) pathway as that for the hyperthermia and puromycin treatments.

If the 5-azacytidine treatment had caused random hypomethylation of DNA, it might have been expected to have resulted in mutations *in vivo*. If mutations were induced by the 5-azacytidine treatment, they were not of a dominant nature as chimaeras and F_1 ES cell-derived progeny were all normal and healthy. However, as the F_1 progeny were not intercrossed, the possibility of recessive mutations cannot be excluded. It could also be possible that any gene sequences that were perturbed by the 5-azacytidine treatment were modified *in vivo* to the correct methylation pattern, as has been suggested elsewhere (Frank *et al.*, 1991).

If some form of epigenetic change has occurred during the establishment of ES cells derived from embryos exposed to the heat shock, puromycin or 5-azacytidine treatments, these changes must have been of a reversible nature, as under appropriate environmental conditions the stem cells retained the capacity for both *in vitro* and *in vivo* differentiation; that is, the stem cells remain developmentally labile. These perturbation treatments have not produced an epigenetic change which has arrested the cells in an undifferentiated state *in vitro*, since within two passages following the removal of STO feeder cells and DIA/LIF from the medium, heat shock-derived ES cells differentiated into cells with a flattened epithelial-like morphology in monolayer culture. Furthermore, when aggregates of heat shock-derived ES cells were cultured in suspension, they formed cystic embryoid bodies. The most compelling evidence for a fully reversible epigenetic change, comes from the finding that all six of the ES cell lines which were injected into host blastocysts and derived from either heat shock, puromycin or 5-azacytidine treatments, produced somatic chimaeras at a high frequency. More importantly, since EC cells possessing grossly abnormal karyotypes have still retained the capacity to participate in apparently normal embryo development (Illmensee and Croce, 1979), in at least two chimaeras from cell lines derived from each of the three perturbation treatments, the ES cells colonised the germ cell lineage and formed functional gametes. This fact and the data from the limited karyotype analyses completed to date, strongly suggests that the treatments have not "transformed" the cells by altering the chromosomal constitution. Rather, these perturbation treatments may have had a more subtle role in modifying the profile of gene expression and so, increasing the capacity of pluripotent cells within the mouse embryo to be diverted from differentiation and established into culture, as ES cells.

Following blastocyst injection of ES cells, derived from either control embryos or embryos exposed to one of the three perturbation treatments, somatic chimaeras

were formed at a high efficiency compared to other studies (*cf.* Table 5.1 and Evans *et al.*, 1985). This was possibly due to the injection of stem cell lines at a low passage number, which had not accumulated large populations of cells with chromosomal abnormalities. Furthermore, stem cells from each of the four ES cell lines examined for germline transmission in these studies were capable of forming functional gametes in chimaeras. From the male ES cell lines, two classes of germline male chimaeras were observed; those which either partially or absolutely transmitted the ES cell genotype. Those male chimaeras which only transmitted the 129/Sv-*CP* genotype in a small fraction of their sperm, would have arisen in the case were male ES cells were injected into a male host embryo (Robertson, 1986). The two absolute transmitters produced in these studies, where all of their sperm were of the 129/Sv-*CP* genotype, presumably resulted as a consequence of male stem cells being injected into a female host embryo which converted the sex of the resultant animal (Robertson, 1986). From the sex ratio with the 129 $HS_{42}20$ male ES cell line (see Table 5.1) it is estimated that half of the female embryos injected with stem cells were sex converted to phenotypic males. Of these sex converted chimaeras, 90% (9/10) did not breed and on autopsy were shown to be sterile hermaphrodites. This presumably occurred from a low contribution of male ES cells to the female germinal ridge during embryogenesis, which only exerted a mild masculinising influence.

These studies have demonstrated the potential of female ES cells to colonise the female germline (figure 5.7). Although cultured female EC cells have produced germline female chimaeras (Stewart and Mintz, 1981) there are no previous reports of germline transmission from female ES cells; if only because this event has not been assayed extensively (see chapter 1.3.6). Because the female 129 $P_{[25]}4a$ ES cell line tested in this study had populations of cells with a 40 XX and 39 XO chromosome constitution, it is not known which cells produced the stem cell-derived oocyte. XO ES cells (from a male cell line where the Y chromosome had been lost from approximately 50% of the cells) have been shown competent to colonise the germline in female chimaeras (Kuehn *et al.*, 1987).

In summary, these studies show that treatments expected to perturb gene expression increase the efficiency of ES cell isolation, suggesting that the isolation of stem cells may depend upon changes in the pattern of gene expression. ES cell lines derived from embryos exposed to either heat shock, puromycin- or 5-azacytidine-containing medium, have retained the capacity to colonise the germline in some chimaeric mice, suggesting any epigenetic change in culture to be fully reversible *in vivo*. Other methods of modifying gene expression may aid in the understanding of the molecular basis behind the action of these treatments.

CHAPTER 6

EXPRESSION OF CELL SURFACE CARBOHYDRATE ANTIGENS DURING OVINE PRE-IMPLANTATION EMBRYOGENESIS

6.1 INTRODUCTION

During murine embryogenesis, the nature of the carbohydrate chains of glycolipids and glycoproteins at the cell surface undergo a sequence of changes. Anti-carbohydrate antibodies, recognising specific oligosaccharide determinants (antigens), have been utilised to characterise changes in core structure synthesis, branching pattern and terminal chain modification (Pennington *et al.*, 1985). Particular carbohydrate chains have been demonstrated on the cell surface of mouse embryos throughout the pre-implantation period, whereas others may appear in a stage-specific manner. Furthermore, these carbohydrate determinants may subsequently become restricted to certain tissues, however, not necessarily in a lineage-related manner (Kimber, 1990). Nevertheless, some cell surface carbohydrate antigens have provided tissue-specific markers in mouse pre- and post-implantation-stage embryos (reviewed by Kimber, 1990), ES cells and their differentiated derivatives (Brown *et al.*, 1991; Kimber *et al.*, 1991). The expression of some carbohydrate antigens is developmentally regulated, coinciding with differentiative events. Thus, some carbohydrate determinants may have important roles in cell-cell interactions and in mediating cell migration and morphogenesis during embryo development.

The carbohydrate determinants on the cell surface of mouse embryos may be one of four main types: **1)** branched or linear poly-*N*-acetyl lactosamine-containing chains, **2)** fucosylated lactosamine-containing chains, **3)** oligosaccharides related to globoside, or **4)** sialylated glycoconjugates (Kimber, 1990). The two basic carbohydrate families detected on mouse embryos, the lactoseries and the globoseries families, originate from a common precursor, lactosylceramide (Richa and Solter, 1986). The lactoseries carbohydrate chains contain repeated *N*-acetyl lactosamine units which may be one of two isomeric forms: either type I chains, with galactose linked ß(1-3) to *N*-acetyl glucosamine, or type II chains, where galactose is instead in a ß(1-4) linkage to the *N*-acetyl glucosamine unit (Kimber, 1990; see Table 6.2). In the mouse, type I

lactosamine-containing chains were not found on the cell surface of pre-implantation embryos (Pennington *et al.*, 1985), however, a sialylated and fucosylated type I chain determinant has been detected at the compacted morula-stage (Kimber *et al.*, 1991). Type I core structures may be also present on the extra-embryonic visceral endoderm and yolk sac endoderm on days 6.5 and 7.5 *p.c.*, respectively (Pennington *et al.*, 1985). Type II lactosamine-containing chains, identified immunologically with certain human auto-antisera (from cold haemagglutinin disease), may be of either branched (*I*-antigen) or linear (*i*-antigen) type chains (Feizi, 1982; see Table 6.2). Branched carbohydrate chains (detected by anti-*I* antibodies) are present at all stages of pre-implantation development in the mouse, however, following implantation these structures are gradually lost from the primitive ectoderm (Feizi, 1982; Kimber, 1990). Linear chains (detected by anti-*i* antibody) first appear at the time of implantation in the mouse on the surface of visceral and parietal endoderm cells (Pennington *et al.*, 1985).

The globoseries carbohydrate chains are based on terminal modifications of the core globoside structure (*N*-acetyl-galactosamine-ß(1-3)-galactose-α(1-4)-galactose-ß(1-4)-glucose-ceramide; Richa and Solter, 1986). One antigen of this family, SSEA-3 (galactose-ß(1-3)-globoside; see Table 6.2) has been detected on mouse embryos with a monoclonal antibody produced by immunising a rat against four- to eight-cell mouse embryos (Shevinsky *et al.*, 1982). SSEA-3 is present on mouse oocytes and subsequently becomes restricted firstly to the ICM of the blastocyst and then to the primitive endoderm (Shevinsky *et al.*, 1982). During implantation, SSEA-3 is a marker of visceral endoderm (Fox *et al.*, 1984). SSEA-3 is present on human, but not mouse, EC cells (Shevinsky *et al.*, 1982) and it has been suggested that the differentiation of human EC cells and embryos involves the transition from globoseries to lactoseries carbohydrate chains (Kannagi *et al.*, 1983). This is not the case for murine embryos, as both the globoseries SSEA-3 antigen and the lactoseries *I*-antigen are present from the oocyte-stage onwards (Shevinsky *et al.*, 1982; Kimber, 1990). However, during murine embryogenesis, certain tissue types may only express one of these two core carbohydrate structures (reviewed by Kimber, 1990).

The addition of simple sugar residues to terminal *N*-acetyl lactosamine oligosaccharides converts these core carbohydrate structures into many new antigens, including the X- and Y-antigens and antigens of the ABH blood group system (see Table 6.2). The addition of fucose in an α(1-2) linkage to the terminal galactose residue of a lactosamine chain results in the formation of the so-called H-antigen. The A-antigen and the B-antigen are formed when the above fucosylation is followed by the respective addition of either an *N*-acetyl lactosamine unit or a galactose sugar, both in a α(1-3) linkage, to the terminal galactose of the carbohydrate chain (reviewed by

Richa and Solter, 1986). The X-antigen (otherwise known as SSEA-1: stage-specific embryonic antigen-1; Solter and Knowles, 1978) is derived from an α(1-3) fucosylation of *N*-acetyl glucosamine (Gooi *et al.*, 1981). A second fucosylation in an α(1-2) linkage to the terminal galactose converts the determinant into the difucosylated Y-antigen (Abe *et al.*, 1983). A high proportion of lactosamine-containing type II chains are capped with sialylic acid (*N*-acetyl-neuraminic acid) during the first two days of pre-implantation development (Kimber, 1990). These terminal chain modifications depend upon the activities of the various specific galactosyl, sialyl and fucosyl transferase and glycosidase enzymes, which may be regulated by both the control of gene expression and by the competition for mutual substrates (Fenderson *et al.*, 1986; Kimber, 1990).

Neither the A- nor the H-antigen have been demonstrated on the surface of cells during the first eight days of murine embryonic development (Pennington *et al.*, 1985). The B-antigen has been reported to appear at the late morula-stage and is present on the trophectoderm (Kimber, 1990). There appear to be uterine factors responsible for the expression of the Y-antigen on the cell surface of eight- to 16-cell embryos and early blastocysts, before the antigenic determinant is synthesised by the embryo itself (Fenderson *et al.*, 1986). Expression of the X-antigen (SSEA-1) occurs from the eight-cell-stage, coinciding with the process of compaction (Solter and Knowles, 1978). The observation that lacto-*N*-fucopentaose III (a milk oligosaccharide with a structure identical to the terminal portion of the X-antigen) decompacted mouse morulae has suggested that the X-antigen may be involved in cell-cell adhesion (Bird and Kimber, 1984). It has been proposed that the X-antigen is involved in secondary stabilisation of interacting membranes, following the initial Ca^{2+} dependent and uvomorulin (cell adhesion molecule) phases of compaction (Kimber, 1988). Determinants carrying the H-antigen on the surface of uterine epithelial cells (Kimber, Lindenberg and Lundblad, 1988) have been implicated in the heterophilic binding to receptors on the trophectoderm in the initial phases of implantation of mouse blastocysts (Lindenberg *et al.*, 1988; Lindenberg, Kimber and Kallin, 1990).

Expression of the X-antigen has been correlated with developmental pluripotency in the mouse; appearing on the cell surface of the ICM, primitive ectoderm, primordial germ cells, EC cells (Fox *et al.*, 1981) and ES cells (Martin and Lock, 1983). However, the X-antigen is weakly expressed on tissues unrelated to the pluripotent lineage and is also found on the cells of the yolk sac, trophoblast giant cells, brain and kidney (Fox *et al.*, 1981; Pennington *et al.*, 1985).

Much is now known in the mouse about the expression of carbohydrate antigens on the embryonic cell surface during early development, although little is understood

about the functional significance of these carbohydrates. The purpose of this study was to examine the temporal and spatial patterns of expression of cell surface carbohydrates on ovine pre-implantation embryos, using anti-carbohydrate antibodies of known specificity. The patterns of antigen expression were investigated over two developmental events; namely, the processes of compaction leading to the formation of the blastocyst and the differentiation of the ICM into the primitive ectoderm and primitive endoderm tissues. An additional objective was to identify tissue-specific markers on the cell surface of early sheep embryos.

6.2 EXPERIMENTAL METHODS

Superovulated Scottish Blackface ewes were artificially inseminated with semen collected from either Scottish Blackface or Welsh Mountain rams. Embryos were surgically recovered from ewes at one of eight embryonic stages, spanning eight days of pre-implantation development; from four day old eight- and 16-cell embryos, 4.5 day old 32-cell morulae, day five compacted morulae, day seven, eight and nine blastocyst-stage embryos, through to day 11 hatched blastodermic vesicles (day 0 is the day of onset of oestrus). Immunohistology was performed on either whole or cryosectioned embryos. Fluorescent labelling of specific carbohydrate antigens was accomplished with a double-layer antibody technique.

6.2.1 Superovulation and Artificial Insemination of Ewes

Oestrus cycle synchronisation of donor ewes was achieved by intravaginal positioning of progestagen sponges (Veramix; each containing 60mg medroxyprogesterone acetate; Upjohn, Crawley, U.K.) for 12 days. Ewes were induced to superovulate with twice daily subcutaneous injections of ovine follicle stimulating hormone (oFSH: Ovagen; Immuno-Chemical Products, Auckland, N.Z.) over four days (0.125 units of oFSH per injection). The progestagen sponge was withdrawn at the time of the seventh oFSH injection. Ovulation was induced with a single intramuscular injection of 0.008mg of gonadotrophin releasing hormone equivalent (GnRH: Receptal; Hoechst Animal Health, Milton Keynes, U.K.) 24 hours after sponge withdrawal. At this time ewes were also tested for oestrus and this was designated day 0 of embryo development.

Fertilisation was accomplished by artificially inseminating super-ovulated donor ewes, with a laparoscopy technique (Evans and Maxwell, 1987) 12-18 hours after the administration of GnRH. Ewes were held in a recumbent position and a local anaesthetic (Lignocaine A: 30mg/ml lignocaine hydrochloride and 0.0125mg/ml

adrenalin; Univet, U.K.) was injected at two points immediately anterior to the udder. Two incisions were then made in the skin and underlying abdominal wall, through which the laparoscope and a glass pipette (5mm diameter; Scotia Glass Technology, Sterling, U.K.) containing the semen, were introduced. 0.2ml of freshly collected semen diluted in phosphate buffered saline (2:1) was injected into the tip of each uterine horn. Following insemination, the skin incisions were closed with (18mm) Martin Michel clips.

6.2.2 Embryo Recovery

Before surgery, donor ewes were penned indoors without access to feed and water for 24 hours. A surgical plane of anaesthesia was induced by an intravenous injection of 10% (w/v) sodium thiopentone (Intraval; RMB Animal Health, Dagenham, U.K.). The ewes were then placed in a recumbent position and the abdomen was prepared by clipping belly wool. The area was washed with a Savlon disinfectant (7.5% w/v chlorhexidine gluconate; ICI, U.K.) solution, followed by a Hibitane spray (5% w/v chlorhexidine gluconate; ICI, U.K.) used to sterilise the skin. A Magills endotracheal tube was inserted and anaesthesia maintained with an oxygen, nitrous oxide and halothane (Flurothane; ICI, U.K.) mixture, utilising a semi-closed circuit system.

A mid-ventral laparotomy was performed under aseptic conditions by making a mid-line incision just anterior to the udder. The uterine horns, oviducts and ovaries were exteriorised. These organs were kept moist by periodically irrigating with warm medium. The ovaries were inspected to determine the number and site of recently formed *corpora lutea.*

Embryos were recovered by "flushing" media through the reproductive tract ipsilateral to the *corpora lutea.* Four to six day old embryos were recovered by flushing both the oviducts and the uterine horns. The base of the uterine horn wall was punctured, a latex Foley catheter (Ch10; Rusch) inserted and its cuff inflated until the lumen of the uterine horn was occluded. A blunt 18 gauge hypodermic needle was then inserted into the infundibulum and 20ml of warm (37°C) phosphate buffered saline supplemented with 0.0036% (w/v) sodium pyruvate, 0.1% (w/v) D-glucose, 0.4% (w/v) bovine serum albumin, 0.0025% (w/v) kanamycin sulphate and phenol red (Ovum Culture Medium: Flow Laboratories) was injected through the oviduct and the uterus and was collected via the catheter, into sterilised concave glass dishes (5cm diameter). Embryonic stages from day seven onwards were recovered by only flushing the uterine horns. A Foley catheter was inserted into the base of the uterine horn as described above. For day 10 and 11 embryos, a size Ch12 Foley catheter

(Rusch) was used. A blunt 18 gauge hypodermic needle was inserted into the tip of the uterine horn, near the utero-tubal junction and 20ml of warm (37°C) Ovum Culture Medium was injected into the uterine horn, taking care to occlude the oviduct by grasping it between fingers and thumb. This bolus of medium was gently massaged along the uterine horn and collected into sterilised concave glass dishes via the catheter. The flushings were examined with a binocular dissecting microscope to locate and recover the embryos.

The donor ewe's reproductive tract was returned to the abdominal cavity and the body wall incision was closed with absorbable polyglycolic acid sutures (Dexon 4-metric; Davis-Geck, U.K.). The skin was closed with several (18mm) Martin Michel clips. At the end of the operation the administration of the anaesthetic was discontinued and a 2ml subcutaneous injection of penicillin (Duphapen LA: 150mg/ml procaine penicillin and 112.5mg/ml benzathine penicillin; Duphar Veterinary, U.K.) was administered. During post-operative recovery, the endotracheal tube remained in place until the return of swallowing reflexes.

6.2.3 Anti-Carbohydrate Antibodies

Eleven antibodies of known carbohydrate specificity (see Tables 6.1 and 6.2) were generously provided by Dr. Susan Kimber (Department of Cell and Structural Biology, University of Manchester). All were monoclonal antibodies (mAb), except for two (anti-*I* and anti-*i*) which were auto-antibodies from polyclonal human sera. Of the nine mAbs, seven were of a mouse IgM isotype, one was a mouse IgG and one was a rat IgM (Table 6.1). The mAbs were provided as hybridoma supernatants, with an approximate concentration of 100μg immunoglobulin/ml.

The mAbs used in this study were specific for carbohydrate antigens which could be divided into four families (see Table 6.1). Antibodies were used to immunostain sheep embryos which recognised branched (anti-*I*) and linear (anti-*i*) type II lactosamine-containing chains and linear type I lactosamine-containing chains (MC2303). A number of mAbs were used which were known to recognise various fucosylated lactosamine antigens, including the H-antigen (667/9E9: specific for type I chains), the X-antigen (630/7H1), the Y-antigen (shared by the mAbs H001, H004 and AH6) and the B-antigen (B006). One mAb (19.9) was known to be specific for lacto-N-fucopentaose II chains capped with sialylic acid. And anti-SSEA-3 antibody was known to bind to a globoside-related determinant. The actual carbohydrate structures recognised by each of these antibodies are illustrated in Table 6.2.

Table 6.1: Antibodies used to detect the expression of oligosaccharide determinants on ovine embryos

CH_2O Family	Antibody Code	Isotype	Specificity *		References
Poly-*N*-Acetyl Lactosamines	anti-*I*	human polyclonal	branched type II chains		Pennington *et al.* 1985
	anti-*i*	human polyclonal	linear type II chains		Pennington *et al.* 1985
	MC2303	murine IgM	LNT type I chains		Brown *et al.* 1991 Lindenberg *et al.* 1988
Fucosylated Lactosamines	667/9E9	murine IgM	LNF I	monofucosylated type I, H-antigen	Lindenberg *et al.* 1988
	630/7H1	murine IgM	LNF III	monofucosylated type II, X-antigen	Lindenberg *et al.* 1988
	AH6	murine IgM	LNnD I	difucosylated type II, Y-antigen	Abe *et al.* 1983
	H001	murine IgM	LND I, LNnD I	difucosylated type I and II chains	Kimber *et al.* 1988
	H004	murine IgM	LNnD I, LNnF I	mono/difucosylated type II chains	Kimber and Lindenberg 1990
	B006	murine IgM	[αGal]	monofucosylated type II, B-antigen	Kimber *et al.* 1988
Sialylated Lactosamines	19.9	murine IgG	Sialylated LNF II	monofucosylated type I chain	Magnani *et al.* 1982
Globoseries	anti-SSEA-3	rat IgM	ßGalactose-Globoside		Richa and Solter 1986

* Refer to Table 6.2 for the carbohydrate structures of the antigenic determinant(s) recognised by each antibody. CH_2O = carbohydrate

Table 6.2: The structure of antigenic determinants recognised by the anti-carbohydrate antibodies used in this study

Antibody Code	Antigenic Determinant(s) Recognised by Each Antibody: Trivial Name	Abbreviation	Carbohydrate Structu
anti-*I*	Branched type II chains		Galß(1-4)*N*AcGlcß(1-6) 1\ Galß(1-4)*N*AcGlc- 2/ (either linkage 1 or 2) Galß(1-4)*N*AcGlcß(1-3)
anti-*i*	Linear type II chains		[Galß(1-4)*N*AcGlcß(1-3)]n
MC2303	Lacto-*N*-tetraose (type I chain)	LNT	Galß(1-3)*N*AcGlcß(1-3)Galß(1-4)Glc
667/9E9	Lacto-*N*-fucopentaose I (H-antigen; type I chain)	LNF I	Galß(1-3)*N*AcGlcß(1-3)Galß(1-4)Glc | α(1-2) Fuc
630/7H1	Lacto-*N*-fucopentaose III (X-antigen; type II chain)	LNF III	Galß(1-4)*N*AcGlcß(1-3)Galß(1-4)Glc | α(1-3) Fuc

AH6	Lacto-*N*-neo-difucohexaose I (Y-antigen; type II chain)	LNnD I	Galß(1-4)*N*AcGlcß(1-3)Galß(1-4)Glc | α(1-2) | α(1-3) Fuc Fuc
H001	Lacto-*N*-neo-difucohexaose I Lacto-*N*-difucohexaose I (^{1} Y-antigen; ^{2} Lewis^b, type I chain)	LNnD I^1 LND I^2	Galß(1-3^2/4^1)*N*AcGlcß(1-3)Galß(1-4)Glc | α(1-2) | α(1-3^1/4^2) Fuc Fuc
H004	Lacto-*N*-neo-difucohexaose I Lacto-*N*-neo-fucopentaose I (+ Y-antigen; - H-antigen, type II chain)	LNnD I (+) LNnF I (-)	Galß(1-4)*N*AcGlcß(1-3)Galß(1-4)Glc | α(1-2) | ± α(1-3) Fuc Fuc
B006	B-antigen	[αGal]	Galα(1-3)Galß(1-4)*N*AcGlc- | α(1-2) Fuc
19.9	Sialylated- Lacto-*N*-fucopentaose II	Sialylated LNF II	*N*AcNeuα(2-3)Galß(1-3)*N*AcGlcß(1-3)Galß(1-4)Glc | α(1-4) Fuc
anti-SSEA-3	ßGal-Globoside		-Galß(1-3)*N*AcGalß(1-3)Galα(1-4)Gal-

Gal = D-Galactose
*N*AcGal = *N*-Acetyl-D-Galactosamine acid
Glc = D-Glucose
*N*AcGlc = *N*-Acetyl-D-Glucosamine
Fuc = L-Fucose
*N*AcNeu = *N*-Acetyl-Neuraminic

Any binding of these primary antibodies to specific cell surface determinants was detected by overlayering a fluorescein isothiocyanate (FITC) conjugated, anti-species second antibody. If bound, the FITC compound fluoresced green when excited with light in the 450-490nm range. The second antibodies (for the anti-human polyvalent immunoglobulins and the anti-mouse IgMs and IgGs) had been raised in the goat (Sigma) except for the anti-rat IgM, which was raised in the rabbit (gift of Dr. S.J. Kimber).

6.2.4 Immunohistology of Whole-Mounted Embryos

This technique was applied for immunostaining cleavage-stage embryos and for the outer trophectodermal staining of blastocyst-stage embryos.

6.2.4.1 Preparation of Embryos

The zona pellucida was removed mechanically from early embryonic stages. This was achieved with the aid of two micromanipulators (Leitz, Germany) mounted on each side of an inverted phase contrast microscope (Nikon Diaphot). Manipulations were performed in a 250µl droplet of medium on a 90mm plastic dish (Sterilin) under paraffin oil. The medium utilised was phosphate buffered saline supplemented with 0.0036% (w/v) sodium pyruvate, 0.1% (w/v) D-glucose and 0.4% (w/v) bovine serum albumin (PB1: Whittingham and Wales, 1969) but without phenol red. While the embryo was immobilised by suction on the tip of a flame-polished holding pipette (external diameter *c.a.* 150µm) a stout, pointed needle, mounted on the opposite manipulator, was used to initially create a tiny tear in a region of the zona adjacent to the holding pipette. It was possible to gradually create a slit around approximately two-thirds the circumference of the zona. By reducing the suction pressure a fraction, the tearing action of the needle slightly rotated the embryo across the orifice of the pipette. This enabled the needle to repeatedly tear a new section of the zona and thus, progress slowly around the embryo. Producing the slit around the zona with expanded blastocyst-stage embryos was facilitated by initially puncturing the blastocoelic cavity, so that the embryo partially collapsed.

The embryo was removed through the large slit created in the zona by gently aspirating the embryo into a mouth-controlled Pasteur pipette, with the glass capillary drawn out so as to have a diameter approximately the same size as the embryo. Zona-free embryos were then ready for immunostaining.

6.2.4.2 Immunostaining Whole Embryos

Zona-free embryos were washed once in PB1 medium (see 6.2.4.1) + 0.02% (w/v) sodium azide, but without phenol red (Lindenberg, Kimber and Kallin, 1990). All antibodies and sera were diluted in this buffer. Embryos were incubated at room temperature for 20 minutes in a 1:20 dilution of normal serum of the species of the second antibody - goat or rabbit serum (Sera-Lab) - to prevent non-specific binding. mAbs were diluted 1:10, except for anti-SSEA-3 (1:20) and a 1:40 dilution was used for anti-*I* and anti-*i* antibodies. Embryos were then introduced by mouth pipette, in the minimum of buffer, into a 25µl drop of the primary antibody under paraffin oil in a clean, solid watchglass and were incubated at room temperature for one hour. Control embryos were incubated in the buffer without primary antibody. Following this, embryos were washed six to eight times in fresh droplets of buffer on the lid of a plastic dish (Sterilin). They were then stained in 25µl droplets of FITC-conjugated second antibody (1:80 dilution) under oil, for one hour at room temperature. The embryos were finally washed six to eight times in buffer before being mounted.

6.2.4.3 Mounting Whole Embryos

Embryos were mounted in optically-true glass microslides (CAMLAB) allowing the embryos, in the buffer, to move up inside the microslide by capillarity. Both ends of the microslides were then sealed carefully with plasticine. Using a drop of Super-Glue (active ingredient: cyanoacrylate), the microslide was fixed to an ordinary glass microscope slide. Embryos were viewed immediately afterwards for fluorescence.

Microslides with different luminal sizes were utilised to accommodate different stage embryos; for example, 0.1mm microslides for eight-cell embryos and up to 0.4mm slides for some day nine blastocysts. Larger blastodermic vesicles were mounted instead in the depressions within tissue-typing slides (BDH) with an overlaid coverslip.

6.2.5 Immunohistology of Cryosectioned Embryos

Blastocyst-stage embryos were sectioned in order to examine the pattern of antigen expression on the cell surface of ICM/primitive ectoderm and primitive endoderm tissues.

6.2.5.1 Preparation of Tissue

Embryos were mounted as quickly as possible after surgical recovery in O.C.T. embedding compound (comprised of water soluble glycols and resins; BDH) contained within 1ml conical cryotubes (Nunc; Life Technologies) which were used as moulds. These cryotubes had been vertically scored on two opposing sides with a hot scalpel blade, to facilitate subsequent opening of the mould. A small drop of the O.C.T. compound was placed at the bottom of the cryotube, taking care not to trap air bubbles. A mouth-controlled, finely pulled Pasteur pipette was then used to introduce the embryos (in the absolute minimal volume of PB1 medium; without phenol red) into the O.C.T., positioning the (one to four) embryos as closely as possible to the bottom of the tube. Working quickly, the embryos were snap-frozen in iso-pentane (BDH), pre-cooled on liquid nitrogen and stored at -80°C until required for sectioning.

6.2.5.2 Cryosectioning

When required, the cryotube was transferred into the cabinet of the cryostat (Brights; Huntingdon, U.K.), set at -15°C. Using two haemastats, the plastic cryotube was broken and the frozen O.C.T. plug, containing the embryos at (or near) the apex, was fixed onto the chuck of the cryostat with a drop of O.C.T.. Sections were cut 7-10μm in thickness. The cut section, lying on the cold knife, was picked up with a clean, warm, glass microscope slide by gently pressing the slide evenly against the knife. To improve adherence of the tissue to the glass, the slides had been dipped into a solution containing 0.1% (w/v) gelatin and 0.01% (w/v) chromium potassium sulphate in distilled water and allowed to air-dry before use (Goldman, 1968). The embryo sections were allowed to dry at room temperature on the glass slides before being fixed for 10 minutes in acetone at 4°C. They were then ready for immunostaining.

6.2.5.3 Immunostaining Cryosectioned Embryos

Fixed sections were allowed to dry at room temperature before being hydrated for three to five minutes in phosphate buffered saline (with Ca^{2+} and Mg^{2+}) containing 0.1% (w/v) bovine serum albumin and 0.05% (v/v) Tween-20 (Sigma) (pH 7.2: Kimber, Lindenberg and Lundblad, 1988). All antibodies and sera were diluted in this buffer. The sections were incubated at room temperature for 30 minutes in a 1:20 dilution of normal serum of the species of the second antibody - goat

or rabbit serum (Sera-Lab). The primary antibodies were diluted in the buffer to the same ratios as described in section 6.2.4.2. After draining the serum and drying around the sections with paper tissues, 10µl of the diluted primary antibody solution was placed over the sections and incubated overnight in a humidified chamber at 4°C. Controls, used to assess non-specific binding, involved the incubation of some sections in the buffer without any antibody. Only one section per embryo was tested with each antibody. Following the overnight incubation, the sections were rinsed four to five times in buffer and then incubated at room temperature for 10 minutes in a 1:20 dilution of normal goat serum. They were then stained in a 1:80 dilution of FITC-conjugated second antibody for one hour at room temperature. The sections were then finally rinsed several times in buffer and mounted in 0.25% (w/v) 1,4-diazobicyclo-(2,2,2)-octane (DABCO; Sigma) in 90% (v/v) glycerol in phosphate buffered saline (pH 8.6; stored at -20°C), to help prevent fading of fluorescence (Johnson *et al.*, 1982). The coverslips were then sealed with nail varnish.

6.2.6 Epi-fluorescence Microscopy

Immunostained sections and embryos were viewed in a Leitz Ortholux epi-fluorescence microscope with a mercury lamp for illumination and excitation filter BP 450-490 (*i.e.* blue light). Fluortar (x25 and x50) water immersion objective lenses were used. Specimens were viewed the day of preparation. Fading was a particular problem associated with whole-mounted embryos and thus, it was important not to expose the embryos to the light beam for too long. A three grade system was adopted to quantify the level of antigen expression on the cell surface as detected by antibody binding. Both brightfield and epi-fluorescence photographs were taken on Tmax 400 ASA film (Kodak). Epi-fluorescence exposure times were between 15-30 seconds for whole-mounted embryos and 30-60 seconds for sectioned material.

6.3 RESULTS

The panel of antibodies used in this study have demonstrated four different patterns of antigen expression during ovine pre-implantation embryo development. Antigens were either **(1)** present on the surface of all cell types at all of the embryonic stages examined or **(2)** were not expressed at all; alternatively other antigens were either **(3)** present during early cleavage-stages and then subsequently became restricted to specific cell types after differentiation or **(4)** first appeared on a specific cell type at a particular stage after differentiation had occurred. A summary of developmentally

n = embryos; only one section per blastocyst-stage embryo was stained with each antibody.

regulated antigen expression during ovine embryogenesis (patterns 3 and 4) is presented in Table 6.3.

Antigens containing branched (*I*) and linear (*i*) type II poly-*N*-acetyl lactosamine oligosaccharides and the globoside-related SSEA-3 determinant (see Tables 6.1 and 6.2) were strongly detected at all developmental stages studied, from eight-cell embryos through to the day 11 blastodermic vesicles and on all tissues; that is, trophectoderm, ICM/primitive ectoderm and primitive endoderm. Between two and 10 embryos or cryosections were stained per embryo stage with the anti-*I*, anti-*i* and anti-SSEA-3 antibodies and controls were negative in all instances.

The pattern of expression of the *I*-antigen is shown in figure 6.1. The *I*-antigen was detected uniformly over the cell surface of each blastomere from at least the eight-cell-stage (n=2) onwards (figure 6.1 a,b). Higher magnification (x500) of morula-stage embryos (n=5) showed the punctate staining pattern of the anti-*I* antibody on the cell surface (figure 6.1 c,d). The *I*-antigen appeared to be relatively more abundant on the trophectodermal cells of blastocyst-stage embryos (n=10), than at earlier cleavage-stages (figure 6.1 e,f). The immunostaining of sectioned embryos demonstrated the expression of the *I*-antigen on the surface of cells of the ICM from day seven and day nine blastocyst-stages (n=6) (figure 6.1 g). Following differentiation of the ICM, the *I*-antigen was present on the surface of both primitive ectodermal and primitive endodermal cells of the so-called "embryonic disc" in the day 11 embryo (n=2) (figure 6.1 h). The anti-*I* antibody also bound strongly to cytoplasmic components in all tissues exposed by the sectioning procedure (figure 6.1 g,h).

The mAb 630/7H1, which recognises the X-antigen in the mouse (see table 6.2), did not bind to the cell surface of ovine embryos, at any of the eight developmental stages which were studied. Between two and five embryos or sections per embryonic stage were examined with 630/7H1. The mAb MC2303, which recognises type I oligosaccharide chains (see Table 6.2), also did not bind to sheep embryos. No staining with MC2303 was detected at the 16-cell-stage (n=2), nor on day nine hatched blastocysts (n=3) or on the trophectoderm, primitive ectoderm and primitive endoderm of day 11 blastodermic vesicles (n=2).

Antigens containing type I carbohydrate chains which were modified with either a terminal fucose residue forming the H-antigen (detected by the mAb 667/9E9) or were both fucosylated and sialylated (detected by the mAb 19.9; see Tables 6.1 and 6.2) were expressed only on the trophectoderm of blastocyst-stage embryos at relatively moderate levels (Table 6.3). Neither 667/9E9 nor 19.9 bound to cleavage-stage embryos. The LNF I determinant (recognised by 667/9E9) was not detected during the process of compaction, but was present on the trophectoderm of the

Table 6.3: Stage- and tissue-specific carbohydrate antigen expression during ovine pre-implantation embryogenesis

Embryo Age (day)	4	4	4.5	5	7		9		11		
Embryo Stage	8-cell	16-cell	32-cell morula	compact morula	blastocyst		hatched blastocyst		blastodermic vesicle		
mAb					troph	ICM	troph	ICM	troph	ecto	endo
AH6	-$_{3}$*	-$_{3}$	+or-$_{2/5}$**	+or-$_{2/10}$	+or-$_{4/8}$	-$_{2}$	+$_{10}$	-$_{2}$	+$_{2}$	-$_{2}$	-$_{2}$
H001	NT	-$_{2}$	+or-$_{1/4}$	-$_{5}$	++$_{3}$	-$_{2}$	++$_{5}$	-$_{2}$	+$_{4}$	-$_{3}$	-$_{2}$
H004	-$_{4}$	+$_{6}$	++$_{5}$	++$_{9}$	++$_{3}$	-$_{2}$	+++$_{8}$	-$_{2}$	++$_{2}$	-$_{1}$	-$_{2}$
B006	-$_{5}$	+$_{3}$	+$_{3}$	++$_{3}$	+++$_{6}$	++$_{3}$	+++$_{6}$	+$_{2}$	+++$_{2}$	-$_{2}$	+$_{2}$
667/9E9	-$_{3}$	NT	-$_{1}$	-$_{2}$	+$_{3}$	NT	++$_{5}$	-$_{1}$	++$_{2}$	NT	-$_{2}$
19.9	NT	-$_{3}$	-$_{4}$	-$_{1}$	-$_{2}$	NT	+or-$_{1/5}$	-$_{2}$	++$_{2}$	-$_{2}$	-$_{2}$

+, ++, +++ level of antigen expression as detected by antibody binding

\- negative

NT not tested

* number of embryos examined

** proportion of embryos staining positive

Embryonic tissues:

troph trophectoderm

ICM inner cell mass

ecto primitive ectoderm

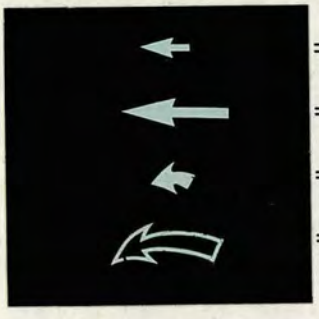
endo primitive endoderm

Figure 6.1: The *I*-antigen is expressed on the surface of all embryonic cells from day four to day 11 of ovine pre-implantation development. The anti-*I* antibodies bound to determinants present on blastomeres from eight-cell embryos (a,b), compacting morulae (c,d) and on the outer trophectoderm of day eight blastocyst-stage sheep embryos (e,f). Immunostaining cryosectioned embryos demonstrated the expression of the *I*-antigen on the trophectoderm and inner cell mass (ICM) of the day nine hatched blastocyst (g) and the trophectoderm, primitive ectoderm and primitive endoderm of the day 11 blastodermic vesicle (h). See the legend below illustrating the arrows identifying the different embryonic tissues.

Photographs were taken using brightfield (a,c,e) or epi-fluorescence (b,d,f,g,h) microscopy.

Whole embryos (a,b,c,d,e,f) and cryosectioned embryos (g,h)

Magnification: x250 (a,b,e,f) or x500 (c,d,g,h)

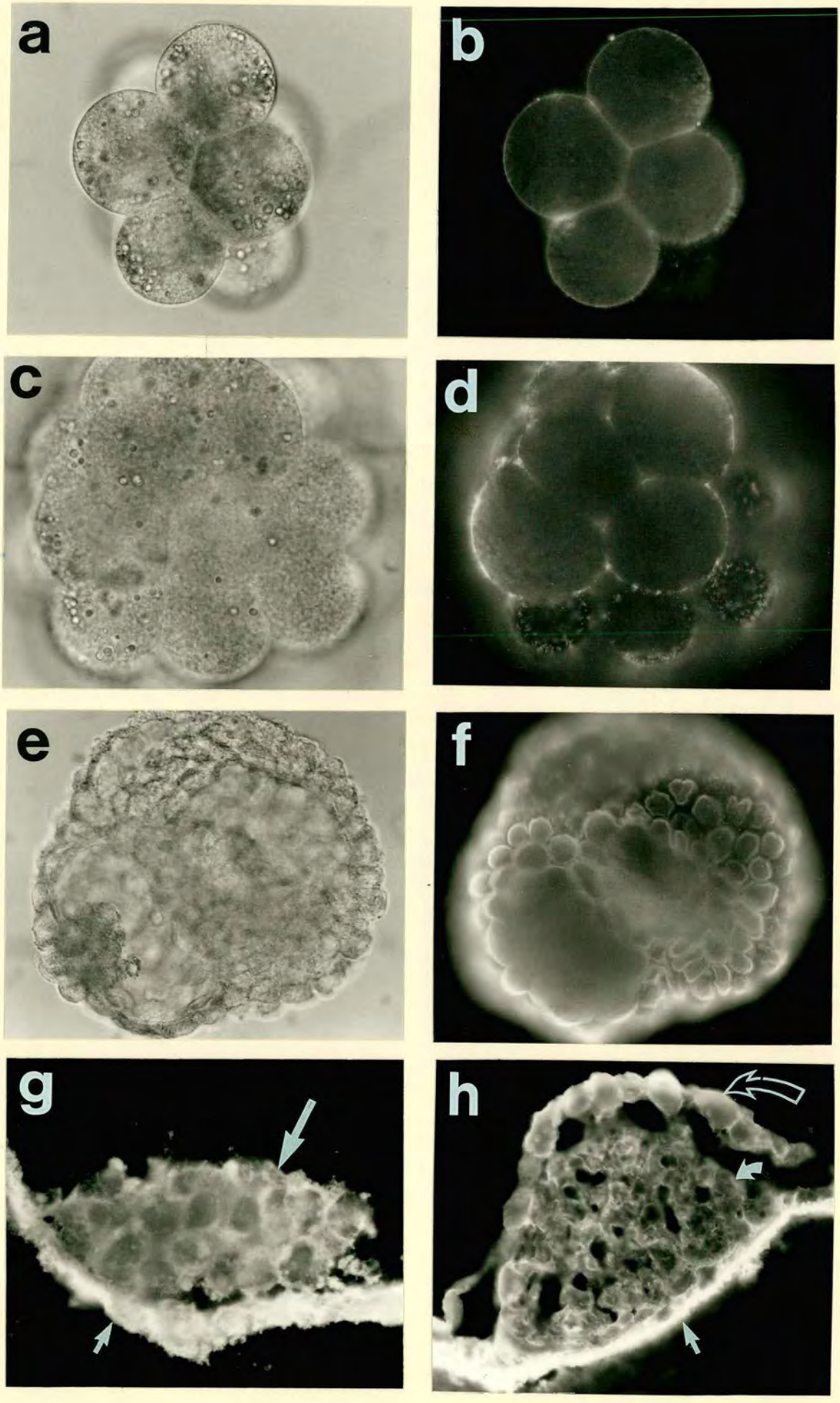


= Trophectoderm

= Inner Cell Mass (ICM)

= Primitive Ectoderm

= Primitive Endoderm


a
b
c
d
e
f
g
h

subsequent blastocyst, at least by day seven. Expression of the sialylated LNF II determinant (recognised by 19.9) was not detected on the trophectoderm until after the blastocyst had hatched from the zona on day nine and then, in only 20% (1/5) of the embryos examined at this stage (Table 6.3). With both 667/9E9 and 19.9, the mAbs tended to bind to the mural trophectoderm in some of the blastocysts studied (3/10 and 1/3 of positive staining blastocysts, respectively), as illustrated for 667/9E9 in figure 6.2a,b. Neither sialylated nor fucosylated type I carbohydrate chain determinants were expressed on the cells of the ICM/primitive ectoderm or primitive endoderm tissues (Table 6.3).

The mAbs AH6, H001 and H004 have in common the recognition of the difucosylated Y-antigen (LNnD I) although they detect this determinant with differing specificity (Tables 6.2 and 6.3). These differences in specificity were demonstrated in the stage at which these antibodies first bound to the cell surface of sheep embryos (Table 6.3). The mAb AH6, which only recognises LNnD I (type II chains), did not bind to embryos until the 32-cell-stage and only bound weakly to some (4/15=27%) morulae. After the first differentiative event, AH6 bound only to the trophectoderm. However, AH6 bound to only half (4/8) of the day seven blastocysts examined. In later developmental stages, the Y-antigen was detected by AH6 on the trophectoderm of the majority (10/12) of eight, nine and 11 day old blastocysts and was not present on other embryonic tissues at these stages (Table 6.3).

The mAb H001, which recognises both difucosylated type I (Lewis^b antigen) and type II (Y-antigen) oligosaccharide chains (Tables 6.1 and 6.2) showed a similar staining pattern as AH6 (Table 6.3). The determinant(s) detected by H001 were not expressed until the 32-cell-stage, although only one of the nine embryos examined stained positive at the morula-stage. After blastocyst formation, H001 bound specifically to the trophectoderm in all embryos examined.

The mAb H004, which recognises a monofucosylated structure (the H-antigen) in addition to the difucosylated Y-antigen (both type II chains; Tables 6.1 and 6.2), bound to embryos from the 12- to 16-cell-stage onwards; not being detected at earlier embryonic stages (Table 6.3; figure 6.3 a-d). The determinant(s) were present on all of the blastomeres in the majority of cleavage-stage embryos studied (16/20=80%). After the formation of the blastocyst, H004 binding was restricted solely to the trophectodermal tissue (Table 6.3; figure 6.3 g,h). In a few blastocysts (2/13), H004 appeared to bind to determinants that were only present over the polar trophectodermal regions (figure 6.3 g). However, this was not the case for the majority of blastocysts, where H004 binding was observed as a discrete, punctate staining pattern over the entire surface of all trophectodermal cells in whole-mounted and

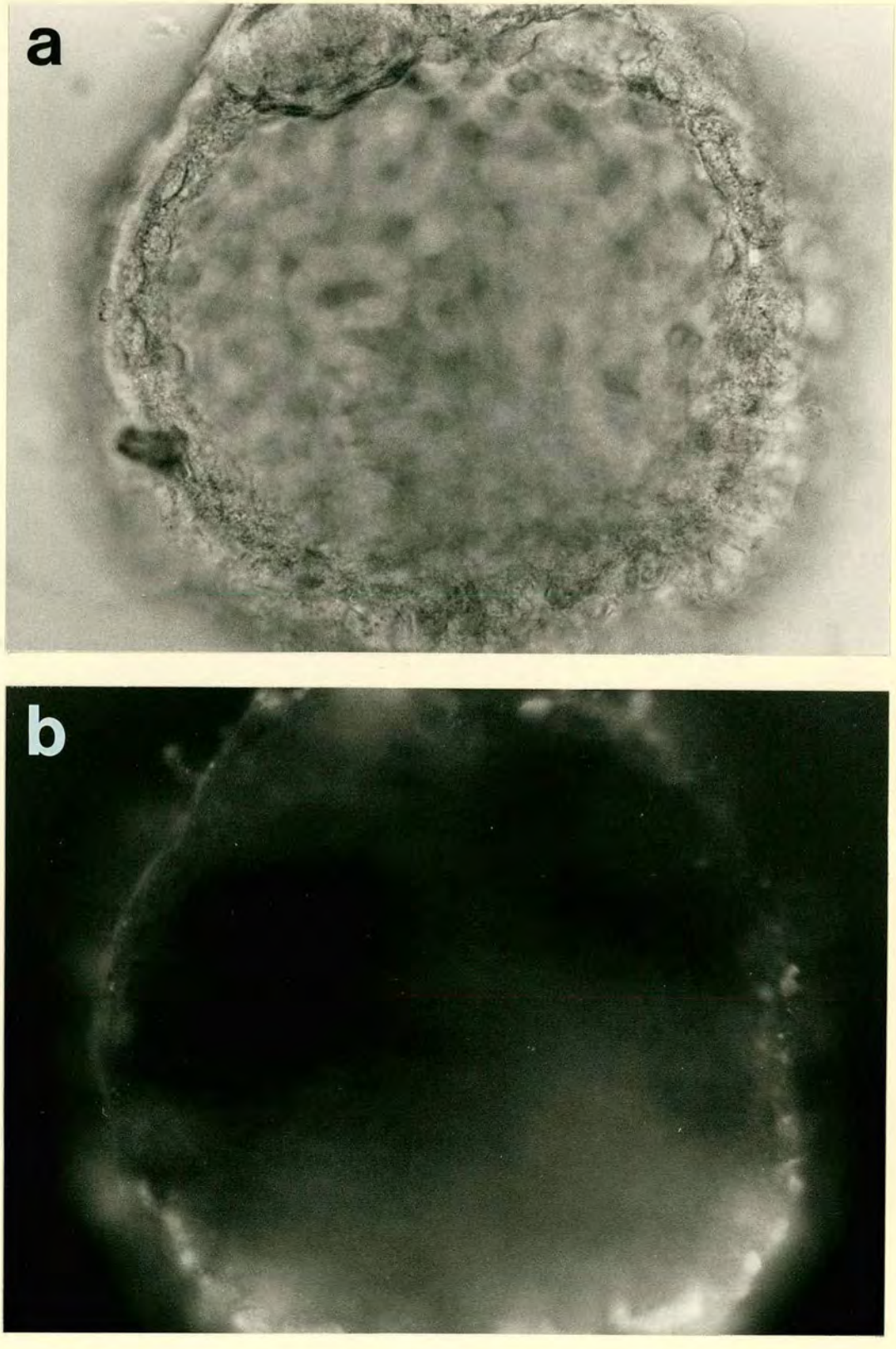


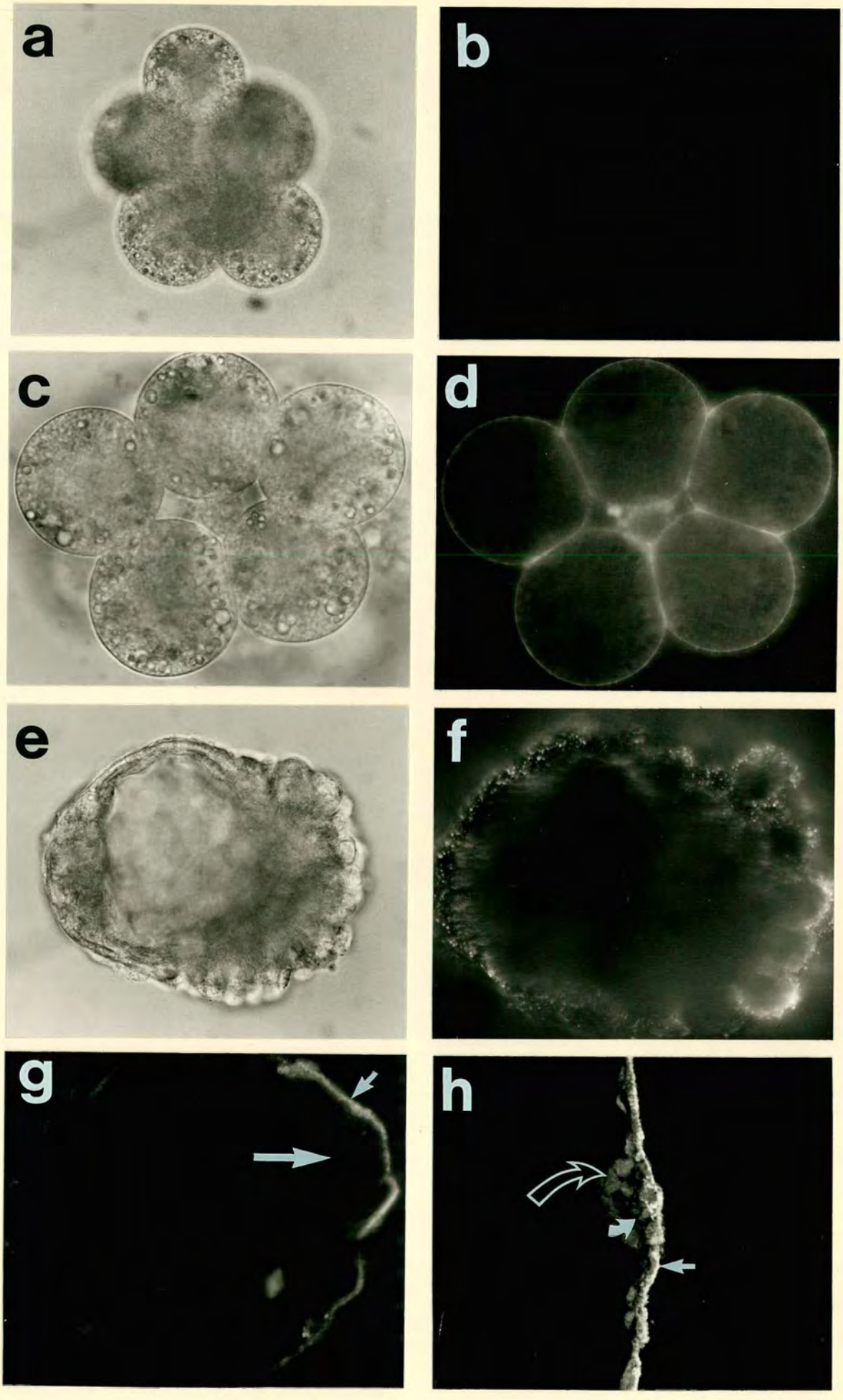
Figure 6.2: Expression of the H-antigen (type I chains), detected by the mAb 667/9E9, on the mural trophectoderm of a day nine sheep blastocyst. Photographs were taken using brightfield (a) or epi-fluorescence (b) microscopy. (Mag.: x250)

Figure 6.3: The antibody H004 detects a determinant(s) with a stage- and tissue-specific pattern of expression on the surface of embryonic cells during ovine pre-implantation development. H004 does not bind to eight-cell embryos (a,b), but does label the blastomeres of 12- to 16-cell embryos (c,d). After blastocyst formation, H004 binds specifically to the trophectoderm in the day seven embryo (g; see the legend below illustrating the arrows identifying the different embryonic tissues). The cryosectioned embryo illustrated in (g) demonstrated a rare instance where only the polar trophectoderm, overlying the negative staining ICM, reacted with H004. In the majority of blastocysts, H004 bound to determinants present over the entire surface of all trophectoderm cells in a discrete, punctate staining pattern; as exemplified in the day nine embryo (e,f). The primitive ectoderm and primitive endoderm tissues of the embryonic disc in the day 11 blastodermic vesicle did not express the antigen(s) recognised by H004 on the trophectoderm (h).

Photographs were taken using brightfield (a,c,e) or epi-fluorescence (b,d,f,g,h) microscopy.

Whole embryos (a,b,c,d,e,f) and cryosectioned embryos (g,h)

Magnification: x250 (a,b,e) or x500 (c,d,f,g,h)


a
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h

cryosectioned embryos (figure 6.3 e,f,h). There was minimal cytoplasmic staining of cells with the mAb H004 in sectioned embryos (figure 6.3 h).

There appeared to be subtle differences in the spatial pattern of antibody staining on the cells of the trophectoderm with the AH6, H001 and H004 antibodies. The antigens detected by H004 appeared to be evenly distributed over the entire cell surface in a discrete punctate pattern (figure 6.3 f,h). The mAb AH6 bound to the Y-antigen which was expressed in a similar punctate staining pattern, but which appeared to be associated slightly more with the intercellular boundaries between trophectodermal cells than H004 (figure 6.4 a). This intercellular pattern of immunostaining was more noticeable with the determinants on the trophectodermal cell surface recognised by the mAb H001 (figure 6.4 b).

The expression of the B-antigen, detected by the mAb B006, was first observed (weakly) on the blastomeres of 16-cell-stage sheep embryos (Table 6.3 and figure 6.5 a,b). The relative level of expression of the B-antigen, as indicated by B006 binding, appeared to increase as cleavage progressed, reaching a maximum on the outer trophectoderm of blastocyst-stage embryos (figure 6.5 c,d). The B-antigen was expressed on cells of the early ICM (figure 6.5 e), however, the intensity of B006 immunostaining decreased on cells of the ICM from more advanced day eight and nine blastocyst-stages (Table 6.3). In the day 11 embryo, the B-antigen was not expressed on the primitive ectodermal cells while at least some cells of the primitive endodermal layer did appear to express this antigen very weakly (figure 6.2 f).

In summary, of the 11 anti-carbohydrate antibodies used in this study, six antibodies bound to determinants which became developmentally restricted on the cell surface of the trophectoderm in the ovine pre-implantation embryo; although one of these six antibodies (B006) recognised the B-antigen which additionally appeared to be weakly expressed on the primitive endoderm.

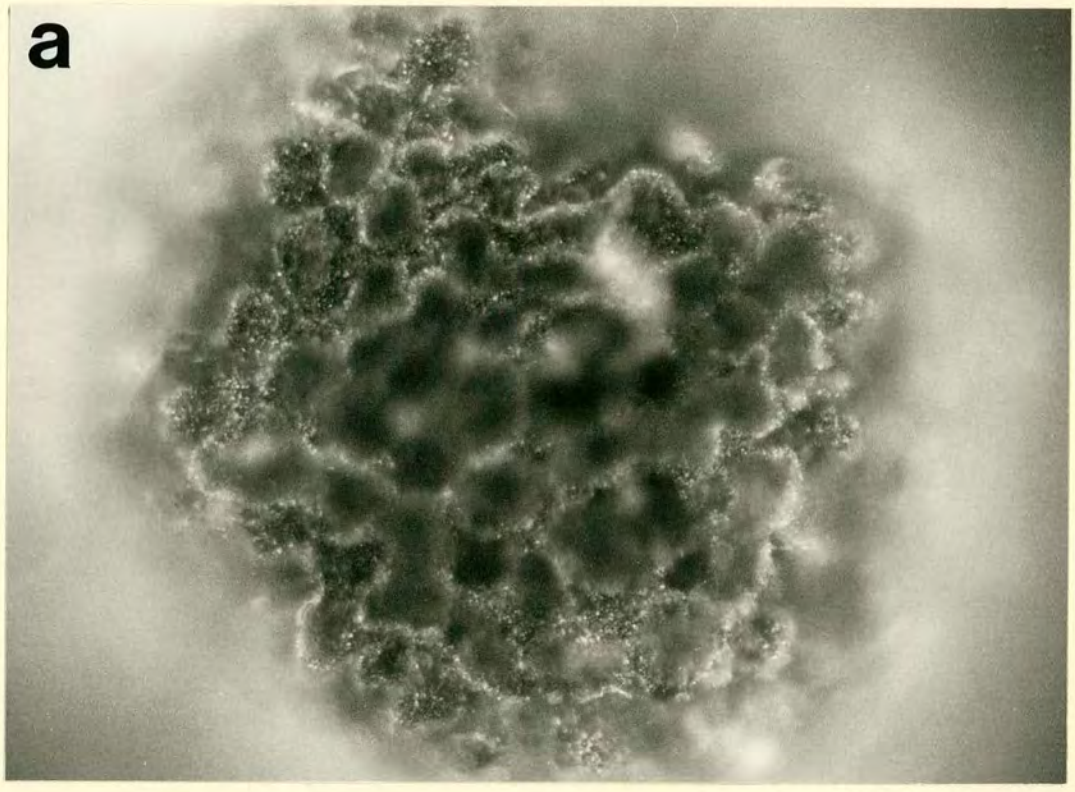
6.4 DISCUSSION

There are a number of similarities and differences between the mouse and the sheep in the profile of carbohydrate antigens expressed during pre-implantation embryo development. With regard firstly to core carbohydrate structures, there is an abundance of branched poly-*N*-acetyl lactosamine (*I*-containing) chains on the cell surface of early ovine embryos, as in the mouse (Pennington *et al.*, 1985). Linear lactosamine (*i*-containing) chains do not appear in the mouse until after implantation (Pennington *et al.*, 1985), however, these structures are present on pre-implantation sheep embryos. As in murine embryogenesis (Kimber, 1990), carbohydrate chains of both the lactoseries and globoseries families are present at all early embryonic stages

Figure 6.4: The spatial patterns of antigen expression detected on the ovine trophectoderm in day eight and day 11 whole embryos by mAbs AH6 (a) and H001 (b), respectively. The Y-antigen (detected by AH6) is expressed in a discrete punctate pattern over the cell surface, but appears to be associated slightly more with intercellular boundaries (a). H001 bound to a determinant(s) expressed predominantly along the intercellular boundaries of trophectodermal cells (b). Note that in the day 11 blastodermic vesicle illustrated in panel (b), the primitive ectoderm of the embryonic disc (arrowed) is exteriorised, no longer having an overlying layer of trophectoderm, and is negative for H001.

Photographs were taken using epi-fluorescence microscopy.

Magnification: x250


a

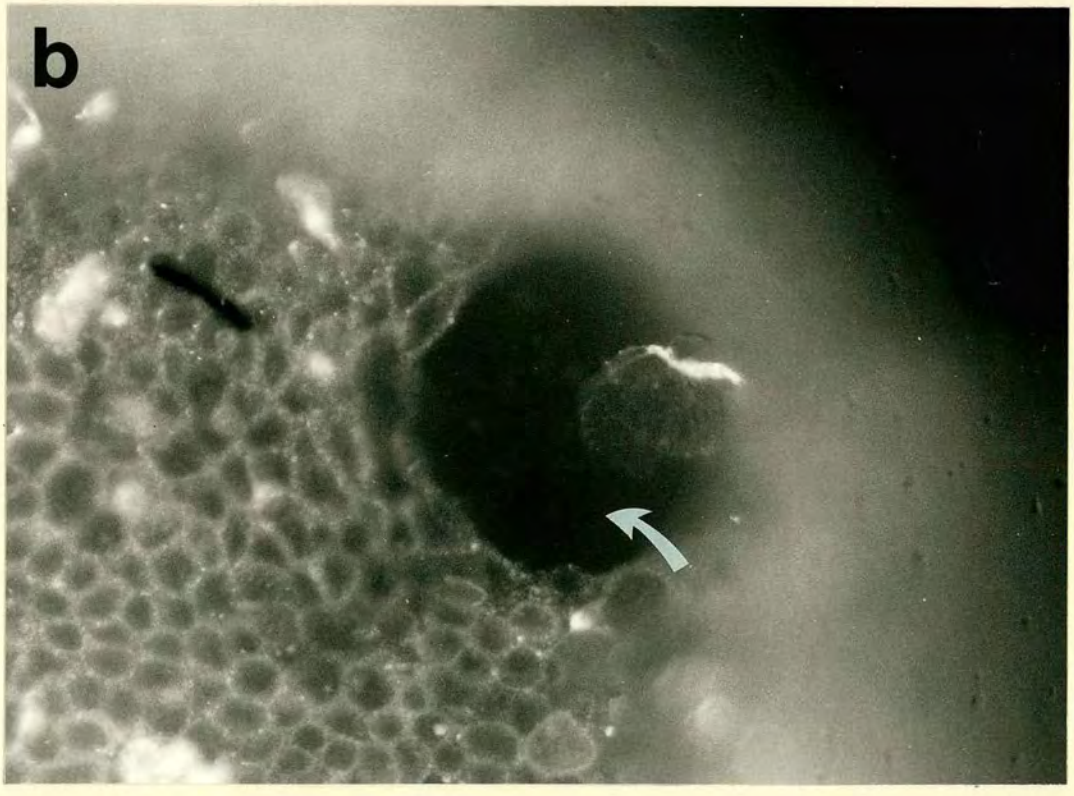
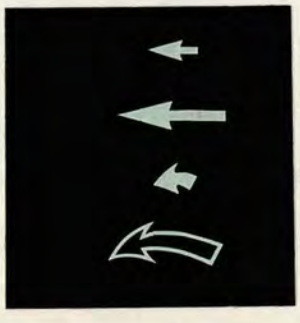

b

Figure 6.5: The B-antigen is expressed in a stage- and tissue-specific manner during ovine pre-implantation embryogenesis. The mAb B006 does not bind to sheep embryos until the 16-cell-stage, on day four of development (a,b). The B-antigen is expressed strongly on the outer trophectoderm of the day nine hatched blastocyst (c,d; panel d shows the upper surface of the embryo in panel c). After blastocyst formation, the B-antigen is expressed on the cells of both the ICM and trophectoderm in the day seven embryo (e; see the legend below illustrating the arrows identifying the different embryonic tissues). As differentiation of the ICM proceeds, the B-antigen is lost from the surface of the primitive ectodermal cells by day 11, although there is evidence of very weak expression on the cell surface of the primitive endoderm at this stage (f). The cryosectioned blastodermic vesicle shown in panel (f) collapsed during the embedding procedure and hence the convoluted nature of the positive staining trophectoderm tissue. Also note that in this embryo, the trophectodermal cells overlying the primitive ectoderm of the embryonic disc have sloughed off by day 11.

Photographs were taken using brightfield (a,c) or epi-fluorescence (b,d,e,f) microscopy.

Whole embryos (a,b,c,d) and cryosectioned embryos (e,f)

Magnification: x250 (c,f) or x500 (a,b,d,e)

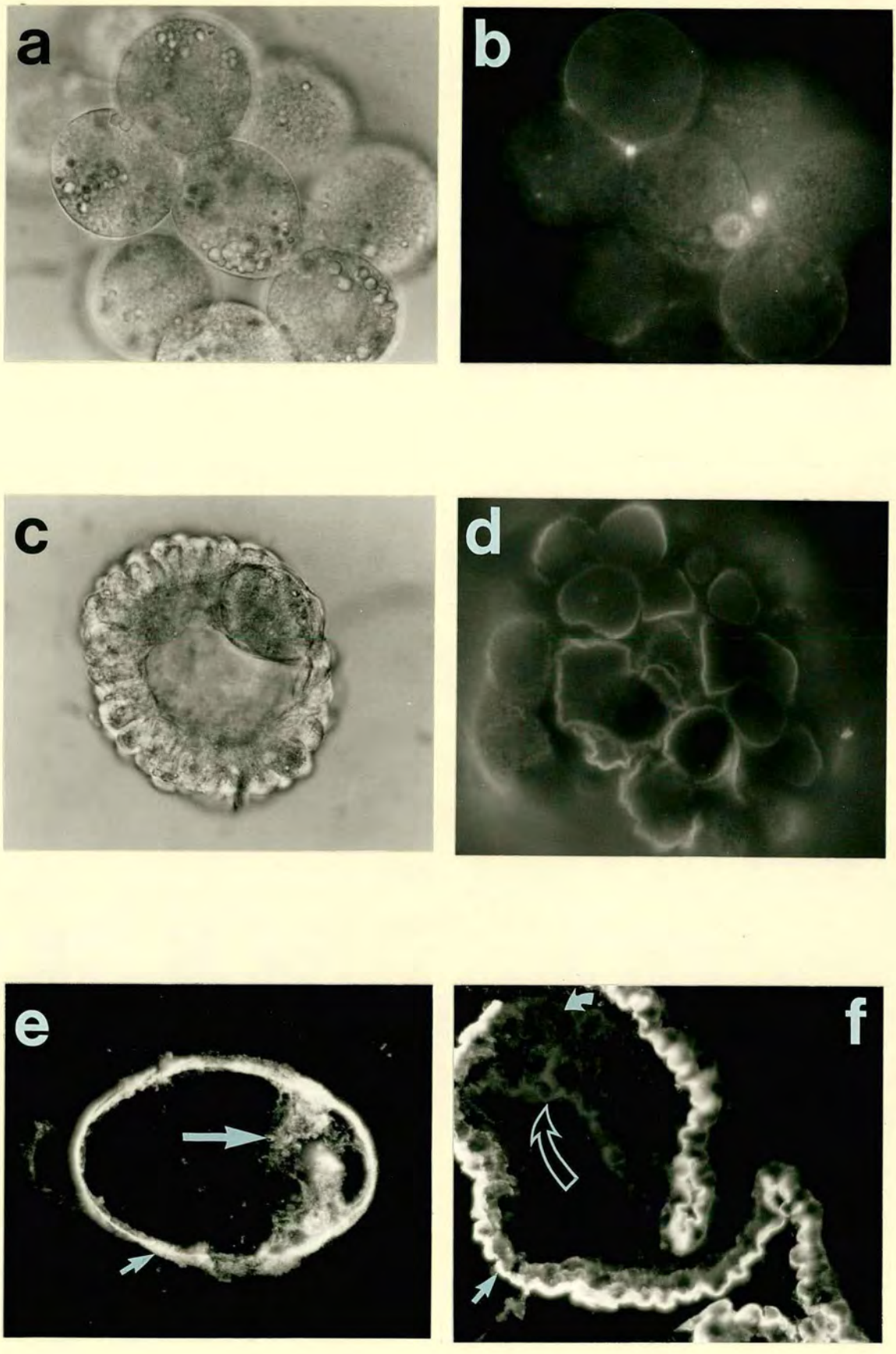


= Trophectoderm

= Inner Cell Mass (ICM)

= Primitive Ectoderm

= Primitive Endoderm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in the sheep. The globoside-related SSEA-3 antigen did not become restricted to the primitive endoderm of ovine embryos, as occurs in the mouse (Shevinsky *et al.*, 1982; Fox *et al.*, 1984).

Unmodified type I lactosamine-containing chains do not appear to be present in either murine (Pennington *et al.*, 1985) or ovine pre-implantation embryos (with no binding with the mAb MC2303). However, two antigenic determinants based on type I core structures, but with terminal chain modifications (detected by mAbs 667/9E9 and 19.9) were expressed in the ewe. The mAb 667/9E9 identifies the monofucosylated, type I chain, H-antigen (LNF I; Lindenberg *et al.*, 1988) which appears specifically on the trophectoderm, after the first embryonic differentiation. The H-antigen (in either type I or type II isomeric forms) has not been demonstrated on the cell surface of pre-implantation mouse embryos (Fenderson *et al.*, 1986; Lindenberg *et al.*, 1988). The sialylated LNF II type I chain determinant, recognised by the mAb 19.9 (Magnani *et al.*, 1982), is first expressed at the time of compaction in the mouse (Kimber *et al.*, 1991) but was not detected in the sheep until after the blastocyst had hatched from the zona pellucida.

Type II lactosamine-containing chains that are terminally-modified with fucose, representative of the Y-antigen and the B-antigen, appear on ovine embryos in a stage-related manner and subsequently become restricted to specific tissues. In the mouse, the B-antigen appears at the compacted morula-stage (Kimber, 1990). In the ewe, however, the B-antigen is first expressed at the 16-cell stage, which is the equivalent of two cleavage divisions earlier than in the mouse. The B-antigen is present on the murine trophectoderm (Kimber, 1990), however, it has not been reported whether the mAb B006 also binds to the murine ICM. B006 did react with the early ovine ICM. Thereafter, the cells of the ICM gradually lost the B-antigen from their surfaces over, at most, four days following the formation of the blastocyst-stage embryo. By day 11, the pluripotent primitive ectoderm of the blastodermic vesicle is negative for the B-antigen, while some of the cells of the primitive endodermal layer, lining the blastocoelic surface of the ectoderm and, by this stage, the trophectoderm also, weakly express this determinant. It is known that the differentiation of the primitive endoderm cells, from the ICM, can occur in some sheep embryos as early as day seven of development (Handyside *et al.*, 1987; McWhir *et al.*, 1991). The primitive endodermal layer may not, however, begin to proliferate rapidly and line the trophectoderm until day 10 (McWhir *et al.*, 1991). The technical limitations of the cryosections obtained in this study from early blastocyst-stages, obscured the presence of primitive endodermal cells delaminated from the ICM and thus, it was difficult to positively determine their antigenicity. In the results, therefore, the endoderm tissue from day nine hatched blastocysts has been grouped with the primitive ectoderm and

termed the "ICM" (see Table 6.3). Although the expression of the B-antigen on the primitive endodermal cells from the day nine sheep embryos is not clear, the results have shown that the B-antigen is lost from the cells of the primitive ectoderm around the time gastrulation occurs in the sheep (McWhir *et al.*, 1991).

The mAb AH6 specifically recognises the difucosylated Y-antigen (LNnD I; Abe *et al.*, 1983). In the mouse, the Y-antigen is first expressed on the blastomeres of uncompacted eight-cell embryos, however, only one-third of embryos appear to stain with AH6 at this stage (Fenderson *et al.*, 1986). Following compaction, the Y-antigen is present on the surface of all pre-implantation embryos in the mouse (Fenderson *et al.*, 1986). This pattern of expression is similar to that in sheep embryos. The Y-antigen (detected by AH6) was stage-specifically expressed just prior to compaction, at the 32-cell stage (but only on 27% of morulae) and did not become expressed on the majority of embryos until the day eight or nine blastocyst-stage. During murine development, the Y-antigen is present on the trophoblast, visceral endoderm and primitive ectoderm of implanted egg cylinder-stage embryos (Fenderson *et al.*, 1986). In the sheep, the pattern of tissue expression of the Y-antigen is very different, as it is only present on the trophectoderm. The antigen appears to be lost from the ICM at the point of blastocyst formation. It would be interesting to section compacted and cavitating morulae to observe if the Y-antigen has been lost from the inner group of cells determined to develop into the ICM. It has been noted in the mouse that the expression of the Y-antigen, up to the blastocyst-stage, appears to be regulated by the uterine environment (Fenderson *et al.*, 1986; Kimber, 1990). Ovine cleavage-stage embryos could be cultured to examine when the expression of the Y-antigen is under embryonic control.

The temporal and spatial patterns of immunostaining detected by the mAbs AH6, H001 and H004 appear to be slightly different compared to each other (Table 6.3; figures 6.3 f and 6.4 a,b). Although all three antibodies have in common the recognition of the Y-antigen, the mAbs H001 and H004 each recognise one other determinant (see Tables 6.1 and 6.2). In addition to the Y-antigen, H001 also recognises the difucosylated Lewis^b, type I chain determinant; structurally classified as LND I (Kimber *et al.*, 1988) and H004 is also known to recognise the monofucosylated (type II chain) H-antigen (LNnF I; Kimber and Lindenberg, 1990). The temporal staining pattern of the H001 antibody is very similar to AH6 (Table 6.3) Although H001 did not bind to compacted morulae, this may have been due to the small number of embryos examined at this stage. In contrast, the H004 antibody bound to determinant(s) possessing a different temporal pattern of expression compared to those detected by AH6 and H001; with H004 binding to the blastomeres of earlier cleavage-stage embryos. There were also subtle differences in the spatial

patterns of immunostaining, in particular the association of staining along the intercellular boundaries of trophectodermal cells, especially with the H001 mAb (and to a lesser extent.with AH6). These differences in the pattern of immunostaining may have arisen as a consequence of the H004 and H001 antibodies each binding to another determinant, in addition to the Y-antigen (specifically recognised by AH6), or they may reflect the subtle differences between the three mAbs in the conformation of the epitope structure required for binding to occur. Subtle changes in the sugars adjacent to the Y-antigen may have influenced the binding specificity and account for the differences observed in the staining pattern between the AH6, H001 and H004 mAbs in ovine embryos and which has also been observed with these three antibodies in ES cells (Brown *et al.*, 1991).

The LNF III, X-antigen (SSEA-1) was not detected at any of the ovine embryonic stages examined. In the mouse this antigen appears at the eight-cell-stage (Solter and Knowles, 1978) and is thought to have a role in stabilising cell-cell interactions during the process of compaction (Bird and Kimber, 1984; Kimber, 1988). None of the antibodies utilised in this studied identified antigens which stage-specifically appeared at the onset of compaction in sheep embryos. Thus, a determinant with a potentially similar function to SSEA-1 was not found. However, in the sheep, the Y-, B-, H- (type I) and the sialylated LNF II antigens all appeared in a stage-specific fashion, although only the H-antigen (type I) appeared immediately after a differentiation event. Furthermore, the above antigens (apart from the B-antigen) eventually became restricted solely to the trophectoderm of blastocyst-stage embryos. Because of these stage- and tissue-specific patterns of expression, it is tempting to postulate that these carbohydrate determinants do have some developmental function. Although the pattern of carbohydrates on the murine embryonic cell surface has been well characterised, a clear understanding of their functional significance is lacking (reviewed by Kimber, 1990). The only embryological processes involving carbohydrate cell surface determinants which have been described in the mouse are blastomere compaction (with the X-antigen; Bird and Kimber, 1984) and implantation, involving heterophilic binding of determinants (the H-antigen) present on the cells of the uterine epithelium (Lindenberg *et al.*, 1990). Although no antigens on the trophectoderm have yet been implicated in murine implantation, perhaps some of the trophectoderm-specific determinants identified here may have a role in the initial phases of implantation in the sheep. One approach in studying the role of these antigens in early embryonic development would be to culture cleavage-stage embryos in medium containing oligosaccharides, to observe whether such treatments perturb normal embryogenesis, with the sugar molecules perhaps binding to receptors on the cell surface.

In the mouse, the X-antigen has been widely used as a marker of the pluripotent cell lineage; being expressed on the ICM, primordial germ cells and ES cells (Fox *et al.*, 1981; Martin and Lock, 1983). None of the antibodies used in this study identified an antigen which was expressed specifically on the cells of the ovine ICM/primitive ectoderm and which could have been used as a potential lineage marker for isolated ovine ES cells. Even in the case of the X-antigen in the mouse, the determinant is also present on tissues unrelated to the pluripotent lineage (Fox *et al.*, 1981; Pennington *et al.*, 1985). This is a disadvantage of using highly specific mAbs, as binding may occur on divergent cell surface molecules, on different cell types, as they may share the same small antigenic determinant (Kimber, 1990). Examination of peri-implantation stages would verify any lineage-related antigen expression. Nevertheless, in this study five anti-carbohydrate antibodies have recognised (at least three) different cell surface determinants present specifically on the trophectoderm of ovine blastocyst-stage embryos, from day seven to at least day 11.

CHAPTER 7

EMBRYONIC STEM CELLS IN THE SHEEP

7.1 INTRODUCTION

Techniques for the isolation and genetic manipulation of ES cells from mouse embryos are now well established (Robertson, 1987; 1991). Because of the interest in extending the stem cell technology to other species of commercial value, some effort has recently been invested from a number of laboratories in attempts to isolate, into culture, embryonic cells possessing a similar developmental potential as murine ES cells from the major farm animal species (see chapter 1.6). Such stem cells would have a major impact on both the livestock industry, opening the way to modifying traits of economic importance, and allowing more fundamental studies on early developmental biology in farm animals.

With specific regard to the ewe, the isolation of only one sheep cell line with a morphology similar to that of murine ES cells has been reported to date, utilising feeder cells, media and general culture techniques similar to those employed for the mouse (Notarianni *et al.*, 1990a; 1991). However, it has not yet been reported whether these sheep cells have the potential to form chimaeras. Other laboratories using similar methods have not been able to maintain colonies, derived from ovine embryos and possessing an ES-like cell morphology (so-called since the pluripotency of this cell type has not been demonstrated) for more than three passages in culture, before the cells either die or differentiate (Handyside *et al.*, 1987; McWhir *et al.*, 1991; Piedrahita *et al.*, 1990b).

The difficulties experienced in isolating stem cells in the sheep may be attributed to two characteristic differences in the pre-implantation embryology between the ovine and murine species. Firstly, the ICM in the ovine embryo is less mitotically active than in the mouse and this is reflected in the slow rates of cell division in sheep embryo cultures (McWhir *et al.*, 1991). Secondly, primitive endoderm differentiation from the ovine ICM commences soon after blastocyst formation on day seven, which is at a relatively earlier developmental stage than the equivalent in the mouse (Gardner and Beddington, 1988; Handyside *et al.*, 1987; McWhir *et al.*, 1991). The presence of endoderm may induce further differentiation of ICM cells in culture (Robertson, 1987) and account for the morphological instability of the undifferentiated ovine cell phenotype *in vitro* (Piedrahita *et al.*, 1990b; McWhir *et al.*, 1991). While it may

appear logical initially to dissect from the embryo pure populations of undifferentiated cells for culture, the immunosurgical isolation of ovine ICMs, free from trophectoderm and endoderm, has not given rise to progressively growing cultures of stem cells (Notarianni *et al.*, 1991). However, in the mouse it has been possible to derive ES cell lines from isolated ICMs (Martin, 1981; Axelrod and Lader, 1983) or primitive ectoderm cells isolated from the post-implantation egg cylinder-stage embryo (see chapter four). Histology has shown the greatest population of undifferentiated cells to be present in the gastrulating day nine to day 10 sheep embryo; a finding which correlated with the observation that ES-like outgrowths were derived at the highest frequencies from these stages (McWhir *et al.*, 1991). So the choice of embryo stage from which to isolate ovine ES cells may be a compromise between the absolute number of ICM/primitive ectodermal cells present and the extent of differentiation. The one sheep ES-like cell line which has been isolated was derived from an outgrowth of an intact day eight embryo (Notarianni *et al.*, 1990a) in which the presence of primitive endoderm on the blastocoelic surface of the ICM would have been expected (Handyside *et al.*, 1987).

In the studies reported in this chapter, embryos from a number of developmental stages, with differing levels of primitive endoderm differentiation, were cultured to determine which gave rise to ES-like colonies at the highest frequency. Embryos from these different developmental stages were cultured in media containing a range of serum concentrations, which may influence cellular differentiation *in vitro*. A serum substitute (Ultroser; Life Technologies) was used in two media formulations to examine the effects on the adhesive properties of embryonic cells. One medium formulation contained the growth factors insulin, which has been shown to preferentially increase the proportion of ICM cells in mouse blastocysts (Harvey and Kaye, 1990) and platelet derived growth factor, added in an attempt to stimulate proliferation of the quiescent ovine ICM/embryonic disc. Ovine embryos were also exposed to heat shock and puromycin treatments, which have been shown to increase the efficiency of ES cell isolation in the mouse (see chapter five).

Cells referred to in this chapter as "ES-like" are defined as those having a high nuclear to cytoplasmic ratio, one of more prominent nucleoli, a relatively small cell size, rounded and possessing no overtly specialised cellular structures. That is, characteristics of a typically undifferentiated cell morphology, as has been described for murine ES cells (Evans and Kaufman, 1981; Martin, 1981) and porcine and ovine ES-like cells (Notarianni *et al.*, 1990a). Classifying colonies as ES-like, is not meant to imply that the cells are pluripotent.

7.2 EXPERIMENTAL METHODS

Ovine embryos for ES cell culture were obtained from three breed combinations; Welsh X Welsh, Welsh X Scottish Blackface and Scottish Blackface X Scottish Blackface. Standard tissue-culture procedures were employed as described in chapter two. The specific treatments examined and the techniques for the culture of ovine embryos from different developmental stages are detailed below.

7.2.1 Stage of Embryo Development

Embryos were recovered from the reproductive tracts of donor ewes on days six, seven, eight and nine (day 0 is the day of onset of oestrus). The procedures for superovulation, artificial insemination and embryo collection from ewes were the same as those described in chapter six (sections 6.2.1 and 6.2.2).

7.2.2 Media Formulation

Ovine embryos were cultured in one of six different media formulations. Media were the same as that employed for mouse ES cell isolation (see chapter two, Table 2.1), except that different serum concentrations and growth factor additions were used. The base media (to which sera and growth factors were added) consisted of Dulbecco's Modification of Eagle's Medium (without sodium pyruvate; Life Technologies) supplemented with 1% (v/v) L-glutamine (Flow Laboratories), 1% (v/v) of a 100X stock of non-essential amino acids (Flow Laboratories), 0.1mM ß-mercaptoethanol (Sigma), with 13% (v/v) Analar water (BDH) added to reduce the osmolarity to around 290mOs. Media did not contain murine or human recombinant DIA/LIF. Hereafter, this base medium shall be referred to as "ES" medium. For sheep ES cell cultures, the sera used were from the same batches of newborn and foetal calf serum as utilised for mouse ES cell cultures. Serum was heated at 56°C for 30 minutes, prior to use, to inactivate the serum complement. In three media formulations, serum was added to give final (v/v) concentrations of 10% (1:1, NCS:FCS; ES_{10}), 15% (1:2, NCS:FCS; ES_{15}) or 20% (1:1, NCS:FCS; ES_{20}) serum.

The serum substitute "Ultroser" (Life Technologies) was used in two media formulations. The lyophilised powder was reconstituted in Analar water to the appropriate volume and was added to ES-medium to give a 4% (v/v) final concentration. In one formulation, the Ultroser was used to completely replace the bovine serum (ES_{US}) and in the second, the medium was supplemented with 5% (v/v) FCS (ES_{US+FCS}). In the sixth medium formulation ($ES_{15+PDGF+I}$), ES_{15} was

supplemented with 20ng/ml of platelet-derived growth factor (PDGF; gift of Dr S. Butterwith, Department of Cellular and Molecular Biology, IAPGR, Roslin) and 10ng/ml of bovine insulin (Sigma).

7.2.3 Heat Shock and Puromycin Treatments

Some day seven to day nine blastocyst-stage embryos were briefly exposed to either an increase in temperature, or to medium containing the antibiotic puromycin, just prior to culture. Ovine embryos were heat shocked for 10 minutes at either 41°C, 42°C, or 43°C; performed as described for the mouse (chapter 5.2.1). For the puromycin treatments, ovine embryos were incubated for 45 minutes in medium containing either 25µg/ml or 75µg/ml of puromycin dihydrochloride (Sigma) (see chapter 5.2.3). Heat shocked and puromycin-treated embryos were subsequently cultured in ES_{15} medium.

7.2.4 Culture of Ovine Embryos for Embryonic Stem Cell Isolation

Sheep embryos were cultured at 38.5°C in a 5% CO_2 in air, humidified atmosphere. Embryos were explanted in groups of between two and six onto mitotically inactivated STO feeder cell layers, prepared in four-well tissue-culture plates (Nunc, Life Technologies) as described previously (chapter 2.1.3). Outgrowths were individually picked out of these group-cultures once they had reached an (empirically determined) suitable stage and gently disaggregated. All the cellular pieces from one embryonic outgrowth were then explanted into a freshly prepared feeder well.

The methods of culture were varied for the different stages of embryo development. Day six morula-stage embryos were disaggregated into individual blastomeres before culture. Zonae pellucidae were removed with an enzymatic solution comprising 0.5% (w/v) pronase (grade B; Calbiochem) and 0.5% (w/v) polyvinylpyrrolidone (M_r 10 000; Sigma) in HEPES buffered "M2" medium (Hogan *et al.*, 1986b). The zona-free morulae were then incubated at 37°C in Ca^{2+} and Mg^{2+} free phosphate buffered saline containing 0.3% (w/v) EDTA (Eistetter, 1989) for three minutes. Dissociation of the blastomeres was completed mechanically utilising a finely pulled Pasteur pipette, with a flame polished tip. All of the cells from each embryo were explanted together into one feeder well.

In some day seven and day eight blastocyst-stage embryos, the ICMs were immunosurgically isolated (Solter and Knowles, 1975). Two anti-sera were utilised to bind to the trophectoderm: the human anti-*I* polyclonal auto-antibodies

(known to bind to sheep embryos, see chapter six; gift of Dr. S.J. Kimber, Department of Cell and Structural Biology, University of Manchester) and heat-inactivated bovine J104 anti-serum (gift of Dr. R.L. Spooner, Department of Immunogenetics, IAPGR, Roslin). J104 is an allogenic anti-serum raised in a cow by a skin graft from its calf and found to be active against sheep lymphocytes (Dr. R.L. Spooner, *pers. comm.*). After an initial 30 minute incubation in either of these two anti-sera (diluted to 1:10) the embryos were washed and then incubated in a 1:5 dilution of guinea pig serum (Sigma) for 45 minutes. Embryos were then gently aspirated repeatedly within a finely pulled Pasteur pipette, to remove the lysed trophectodermal cells. However, since immunosurgery in neither anti-sera was reliable in isolating the ICM, the majority of day seven and eight embryos were cultured intact, but after the zona had been removed with pronase digestion (in those embryos that had not already hatched from the zona).

It has been noted that the majority of intact blastocyst-stage embryos attach to the STO feeder layer by the polar trophectoderm (here and McWhir *et al.*, 1991). Shortly after intact day seven and eight blastocysts had firmly attached to the STO feeders, it was sometimes possible to "peel" away the trophectoderm (which in the process of embryo outgrowth collapsed before growing outwards radially) using sterile watchmakers forceps. This trophectoderm peeling procedure (McWhir *et al.*, 1991) sometimes left the ICM intact and attached to the culture surface, but without any surrounding trophectoderm. After this operation, a small volume of freshly inactivated STO feeders were added into the well to replace the cells removed from around the ICM.

In day nine hatched blastocysts, the embryonic disc (as by convention the ovine ICM is termed from this stage onwards; McWhir *et al.*, 1991) was generally large enough to be dissected mechanically. Working within a still-air cabinet, in the dissecting microscope (magnification *c.a.* x50), two sterile watchmakers forceps were carefully used to tear away the trophectoderm from around the embryonic disc. With practice, very little trophectoderm remained attached to the embryonic disc after the operation. In the case of day nine embryos which were heat shocked, micro-dissection was performed after the hyperthermia treatment.

Although embryos were group-cultured, each outgrowth (derived from either a single blastomere, an intact blastocyst, an immunosurgically isolated or micro-dissected ICM/embryonic disc) was treated individually in decisions regarding when and how to disaggregate the ES-like component. Outgrowths were either disaggregated mechanically or enzymatically. Some outgrowths which grew initially as a monolayer of cells were broken-up mechanically with the aid of a blunt glass probe, constructed from a hand-pulled Pasteur pipette. It was possible to score the

probe across the outgrowth of ES-like cells a number of times to produce several cellular clumps, which were then transferred to a fresh feeder well. Other outgrowths, which instead grew in more upright, three-dimensional colonies, were disaggregated enzymatically in one of three trypsin-based solutions. Some outgrowths were disaggregated in "TEG" (see chapter two, Table 2.2a), however, the majority were disaggregated in a solution designated here as "TED" (Robertson, 1987), or TED with 1% (v/v) chick serum (Sigma) added (Smith and Hooper, 1987). TED is comprised of the same basic salts solution as in TEG (chapter two, Table 2.2a) but contains EDTA (ethylenediamine-tetraacetic acid) instead of EGTA (ethyleneglycol-tetraacetic acid), an alternative source of trypsin (1:250 Difco trypsin; Difco Laboratories; rather than a 10x stock containing 2.5% trypsin in Hank's Balanced Salts Solution, Flow Laboratories) and does not contain polyvinyl alcohol. TEG and TED contain the same concentrations of trypsin (0.25%) and chelating agent (0.04%).

For enzymatic disaggregations, each outgrowth was picked off from the STOs and was transferred through two wash drops of TEG/TED, to remove traces of serum, before incubation in a fresh drop of TEG/TED. With TEG, outgrowths were incubated at 37°C for around five minutes before each outgrowth was finally broken into several cellular pieces (each around 15 cells) by repeatedly aspirating the outgrowth inside a fine, hand-pulled Pasteur pipette. The cells were then passaged into a fresh feeder well. With TED (± chick serum), outgrowths were incubated at room temperature for three to five minutes and disaggregation was completed with gentle mechanical manipulation as described above.

The majority of colonies in the first passage cultures were often of a differentiated cell morphology. If these differentiated colonies threatened to overgrow any small, slowly growing ES-like colonies in the same tissue-culture well, the ES-like colonies, if large enough, were generally picked off the feeders carefully and transferred intact into a fresh feeder well. Alternatively, the differentiated colonies were sometimes removed from the well by peeling them from the gelatin coated tissue-culture surface with a pair of sterile forceps and replacing them with freshly prepared STO feeder cells.

First passage ES-like colonies were generally disaggregated after seven to 10 days of growth, changing the medium regularly throughout this period before it became acidic. Typically, all ES-like colonies derived from an individual embryo were pooled together. The medium in the well was aspirated, the cells washed in PBS (chapter two, Table 2.2b) and then 0.25ml of TEG/TED (± chick serum) was added. Cells were trypsinised at room temperature for three minutes and in the dissecting microscope, a mouth-controlled, finely pulled Pasteur pipette (backfilled

with a small volume of medium) was used to aspirate the ES-like colonies out of the well, just as they began to dissociate from the underlying STOs (leaving behind any differentiated colonies in the process). The aim was to disaggregate these colonies into small clumps of between six to 10 cells. These cellular pieces were then transferred to a fresh feeder well. ES-like cells which survived the trypsinisation procedure and maintained a stable morphology, were generally passaged onto fresh STO feeder wells every seven to 10 days thereafter.

7.2.5 Statistical Analyses

Statistical analyses of the differences between treatments, in the proportions of embryos giving rise to ES-like outgrowths and colonies at each successive passage, was accomplished utilising the chi-squared test (see chapter 2.6 for the mathematical formulae).

7.3 RESULTS

A total of 247 sheep embryos were cultured in studies aimed towards isolating permanent cell lines of ES-like morphology. However, no colonies of ES-like cells were maintained in culture for more than three passages. As there were no differences in the frequency of isolation of ES-like cells from sheep embryos of different genotypes (Welsh X Welsh, Welsh X Scottish Blackface or Scottish Blackface X Scottish Blackface) the data from the three breed combinations have been combined. The results of the effects of stage of embryo development, media formulation, heat shock and puromycin treatments are presented as survival profiles of the percentage of embryos yielding ES-like cell colonies at each passage in culture. Only embryo outgrowths or colonies which maintained a stable ES-like morphology throughout each passage were included in the data.

7.3.1 The Effect of Stage of Embryo Development on the Isolation of Ovine ES-like Cells

The survival profiles of ES-like colonies derived from individual sheep embryos from the four stages of development are plotted in figure 7.1. The graph shows the progressive decline in the proportion of embryos explanted (from all stages) giving rise firstly to ES-like outgrowths and then to the survival and growth of ES-like cells after each successive passage in culture. ES-like colonies derived from day nine embryos were maintained for the longest periods in culture; but not for more than three

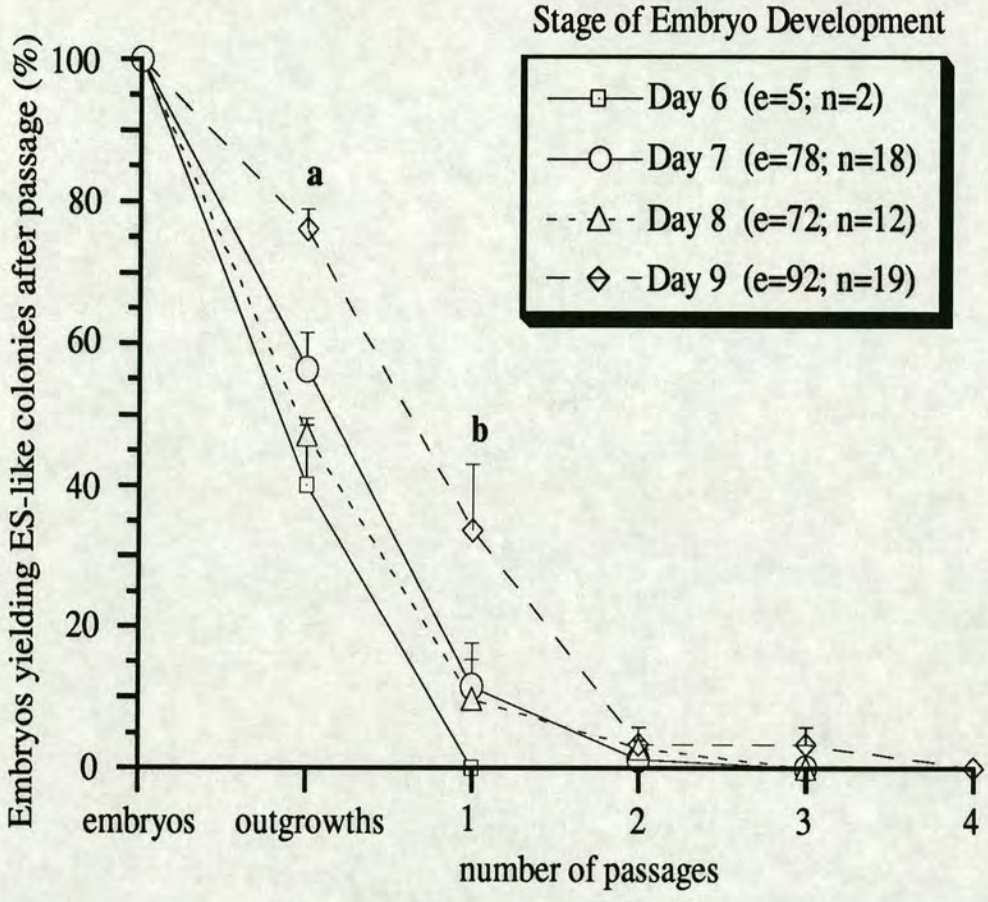


Figure 7.1: The survival profiles showing the effect of stage of embryo development on the percentage of ovine embryos giving rise to ES-like outgrowths and colonies after repeated passage in culture.
(mean ± s.e.m.; e = embryos; n = replicates)
a $P<0.025$ *cf.* day seven and $P<0.001$ *cf.* day eight
b $P<0.005$ *cf.* day seven and $P<0.001$ *cf.* day eight

passages, before they either differentiated or senesced. The data presented in figure 7.1 have been compiled from all embryo treatments in the studies conducted here, as no interactions existed between media, heat shock or puromycin treatments and embryo stage. Day nine embryos consistently gave rise to more ES-like outgrowths and early passage colonies, which tended to maintain an ES-like morphology longer in culture than earlier embryonic stages (figure 7.1). Of the day seven and eight

embryos that were cultured, the ICM was immunosurgically isolated from only around 15% of the blastocysts and because the potential of day seven and eight ICMs and intact blastocysts to give rise to ES-like colonies were not significantly different, the respective ICM and intact blastocyst data have been combined for these two stages.

Day nine embryonic discs gave rise to a significantly greater proportion of ES-like outgrowths (70/92=76.1 ± 2.8%) compared to day seven (44/78=56.4 ± 5.1%; $P<0.025$) and day eight embryos (34/72=47.2 ± 2.2%; $P<0.001$) (figure 7.1). Some of the blastomeres from two of the five day six morulae that were disaggregated attached to the STO feeder layer and divided, to produce small ES-like colonies. The majority of single blastomeres which did attach, however, gave rise instead to trophoblast-like outgrowths.

Day nine micro-dissected embryonic discs and day seven and eight immunosurgically isolated ICMs attached to the STO feeder layer after about 24 hours. Intact blastocysts (with the zona removed) were slower to attach, not beginning to outgrow until two to four days after explantation. A substantial proportion of intact blastocysts failed to attach at all and this loss contributed to the difference in the proportion of ES-like outgrowths between day seven and day eight embryos, compared to day nine embryos.

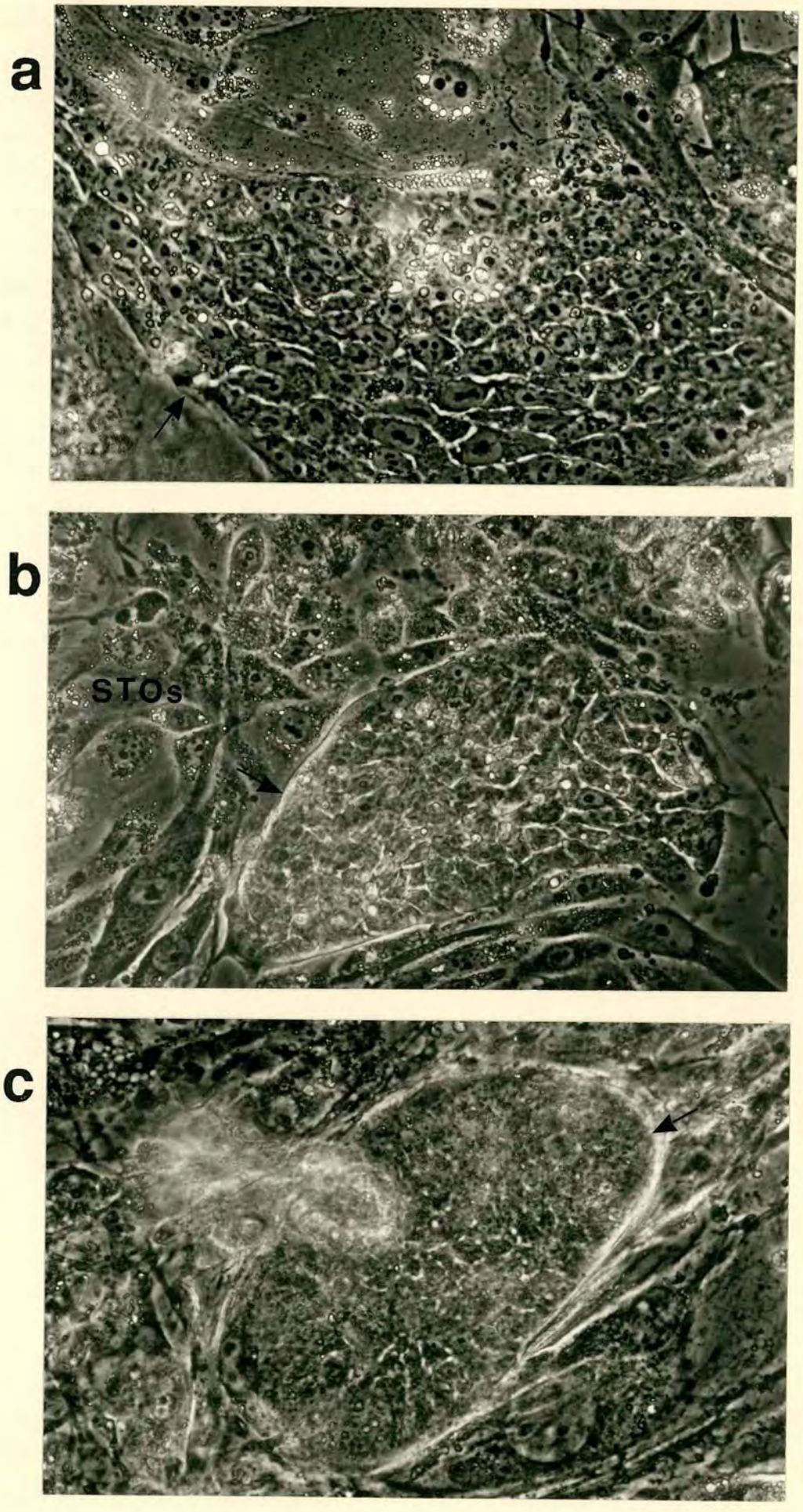
In addition to the higher rates of attachment, day nine embryonic discs also tended to give rise to a higher proportion of outgrowths with an ES-like morphology than earlier embryonic stages. Many intact blastocysts which did attach, failed to produce outgrowths from the ICM and only differentiated cell types were observed. Many blastocysts simply collapsed upon attachment, with the trophectoderm not growing outwards to expose the ICM and trophectodermal peeling was required to reveal the ICM component. Although immunosurgically isolated ICMs from day seven and eight embryos readily attached, few of these small cellular masses resulted in proliferating outgrowths. Most larger day nine embryonic discs gave rise to monolayer outgrowths of cells with a typical ES-like cell morphology, although the cells appeared large (figure 7.2 a). Other embryonic discs resulted in outgrowths comprised of tightly packed "nests" of cells which tended to grow in three-dimensions, without any obvious signs of differentiation (figure 7.2 b).

Cultures were monitored daily and outgrowths derived from day nine embryonic discs with an ES-like morphology were passaged over a four to 10 day interval after explantation. Outgrowths containing different numbers of cells were disaggregrated systematically over this period. Outgrowths containing less than around 50 cells were not passaged; however, outgrowths were disaggregated before they had begun to differentiate extensively. Within this range, there did not appear to be a consistent correlation between the cell number of the outgrowth and the

Figure 7.2: Morphology of ovine ES-like cells cultured on STO feeder layers. Panels (a) and (b) show outgrowths of ES-like cells (see arrows) derived from dissected day nine embryonic discs after four days of culture. Most embryonic discs gave rise to monolayer outgrowths of ES-like cell morphology (a). Although individual cells were relatively large and there were intercellular spaces between the cells of the outgrowth, each cell did possess a high nuclear to cytoplasmic ratio and one or more prominent nucleoli (a). Other day nine embryonic discs gave rise to outgrowths which grew as tight "nests" of cells, with clear clonal boundaries (b). After two passages in culture, discrete colonies of ES-like cells were observed in cultures derived from a small proportion of day nine sheep embryos (c).

Photographs were taken using phase contrast microscopy.

Magnification: x200


a
b
STOs
c

subsequent development of ES-like colonies at the first passage. Monolayer outgrowths were generally disaggregated mechanically, while the more three-dimensional outgrowths were disaggregated enzymatically. Outgrowths derived from day seven and day eight intact blastocysts were generally disaggregated seven to 12 days after explantation; with initial culture being extended slightly due to the delay in embryo attachment to the STO feeder cells. Any outgrowths from which only differentiated cell types had been observed throughout the culture period (which were not included in the data in figure 7.1), were also disaggregated after about 10 days. No discrete colonies of ES-like morphology resulted in the first passage cultures derived from these outgrowths.

There appeared to be a direct relationship between the proportion of ES-like outgrowths and the proportion of embryos giving rise to first passage ES-like colonies (figure 7.1). The disaggregation of day nine outgrowths resulted in a significantly higher proportion of ES-like colonies at the first passage (31/92=33.7 ± 9.3%) compared to either day seven (9/78=11.5 ± 6.1%; $P<0.005$) or day eight embryonic outgrowths (7/72=9.7 ± 5.5%; $P<0.001$). No ES-like colonies resulted from disaggregated outgrowths derived from individual day six blastomeres (figure 7.1).

The losses in the proportion of ES-like cells in the transition from embryo outgrowth to the first passage were largely due to differentiation. Many disaggregated outgrowths only gave rise to differentiated cell types or alternatively, colonies which were initially ES-like gradually altered their cell morphology over several days (only colonies which maintained a stable ES-like morphology throughout each passage in culture were included in the data). Some additional losses of ES-like outgrowths were due to the failure of any of the cells from a particular outgrowth to survive the trypsinisation protocol, regardless of whether chick serum was present in the trypsin solution. (At least some cells survived to the first passage from all outgrowths disaggregated mechanically). TED was more effective than TEG in disaggregating outgrowths. The Difco trypsin in the TED solution appeared to be more biologically active in dissociating the embryonic cells. Furthermore, incubations in TED were able to be performed at room temperature and additionally, required a substantially shorter period than TEG to cause the desired extent of cellular dissociation before serum-containing medium was added and disaggregation completed mechanically.

At the second passage, there were no longer any differences between the day seven, eight and nine blastocyst-stage embryos in the proportions giving rise to ES-like colonies (1.3 ± 1.0%, 2.8 ± 1.1% and 3.3 ± 2.5%, respectively; figure 7.1). Some of the colonies present at the second passage possessed a classical ES-

like cell morphology (figure 7.2 c). However, only ES-like colonies derived from day nine embryos survived to the third passage (3.3 ± 2.5%), but could not be maintained any longer in culture (figure 7.1). Colonies derived from day nine embryos appeared to show relatively faster rates of cell division initially and for a more prolonged period, than earlier embryonic stages. The ES-like colonies derived from day nine embryos eventually behaved in a similar fashion to those of earlier embryo stages, however and reached a point were cell division began to slow down markedly (occurring around the third passage), resulting in poor cell survival after trypsinisation. The ES-like cells also began to senesce around this time, with any surviving cells having differentiated by the fourth passage.

A common pathway of differentiation of early ES-like colonies was to a more epithelial-like morphology. This change in cell morphology is illustrated in figure 7.3 a-c; where the cells of an ES-like first passage colony (a) appeared to rapidly enlarge in size 12 hours later (b) and had differentiated into large, cuboidally-shaped cells possessing large intercellular spaces after the second passage (c). These epithelial-like cells tended to grow directly on the gelatin-coated tissue-culture plastic surface, pushing the STO cells to the periphery of the colony (figure 7.3 c). A number of epithelial-like colonies derived from day nine embryos were expanded and maintained as cell lines for at least 10 passages in a variety of different media, without STO feeder cells; that is, with the cells growing directly on the gelatin. The one epithelial-like cell line that was examined, could not be induced to differentiate *in vitro* in suspension culture.

Other cell types commonly observed in embryo cultures included trophoblast-, endoderm-, fibroblast- and neuronal-like cells. A number of sheep fibroblast-like cell lines, derived from day seven and nine embryos, have also been isolated and maintained without feeder cells. Some cell lines are stable, at least up to the tenth passage, however, other fibroblast-like cell lines gradually differentiated over several passages into more cuboidally-shaped cells.

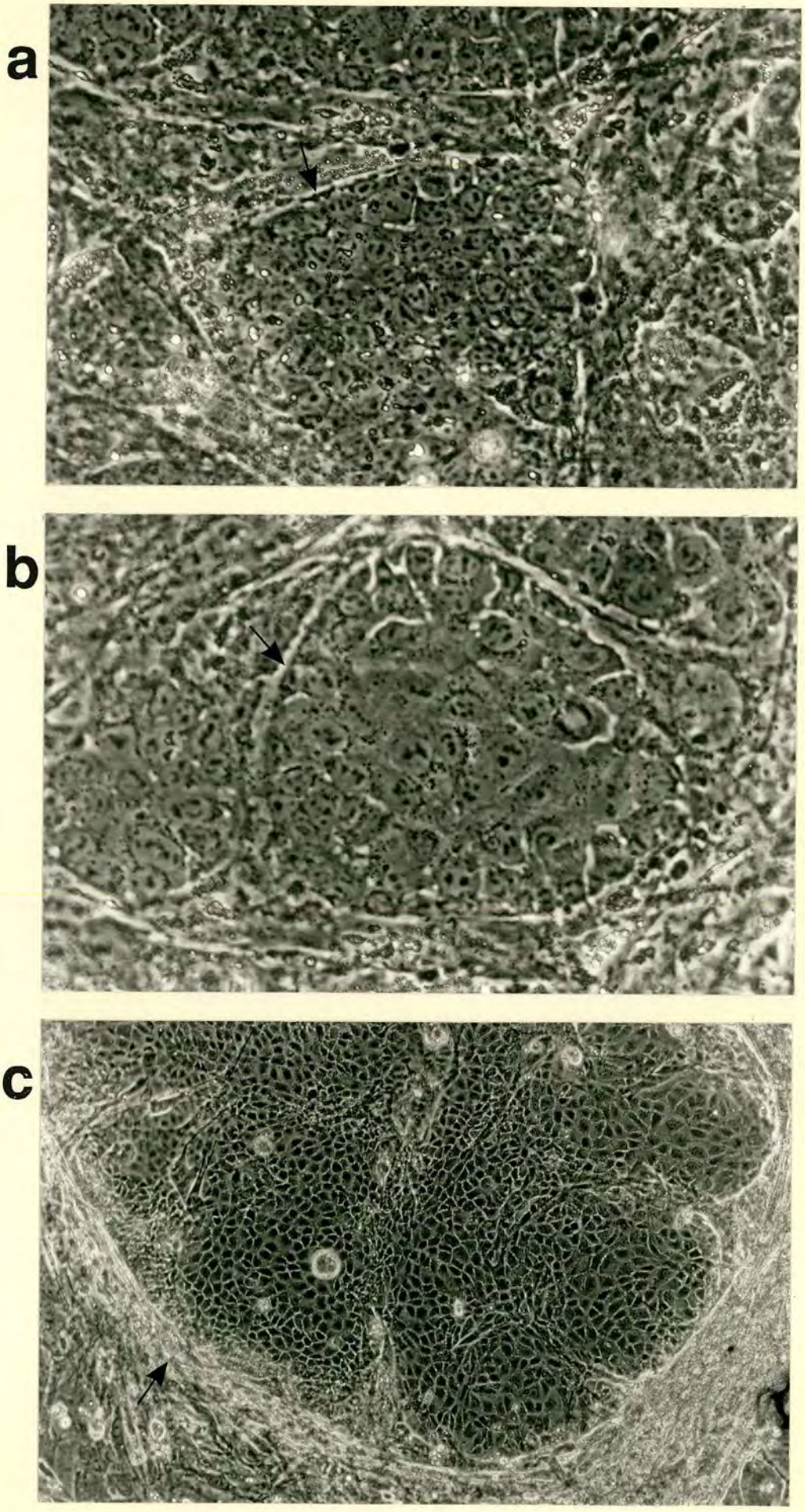
7.3.2 The Effect of Media Formulation on the Isolation of Ovine ES-like Cells

Some day seven, eight and nine blastocyst-stage embryos and the respective cell colonies derived from them, were cultured in a variety of media; to examine the effects of different serum concentrations, a serum substitute (Ultroser) and, growth factors, on the capacity of embryos to give rise to ES-like colonies and their subsequent maintenance *in vitro*. The results are presented in figure 7.4 as survival profiles showing the proportion of day seven, eight and nine embryos

Figure 7.3: Morphological instability of the ovine ES-like cell phenotype. The cells of a small first passage ES-like colony (arrow in panel a) began to enlarge markedly in size 12 hours later (b). When this colony was subsequently disaggregated, the cells differentiated into cuboidally-shaped epithelial-like cells at the second passage (c). These epithelial-like colonies tended to grow directly on the gelatin-coated growth surface, pushing the STO feeder cells to the periphery of the colony (arrow in panel c).

Photographs were taken using phase contrast microscopy.

Magnification: (a,b) x200 or (c) x100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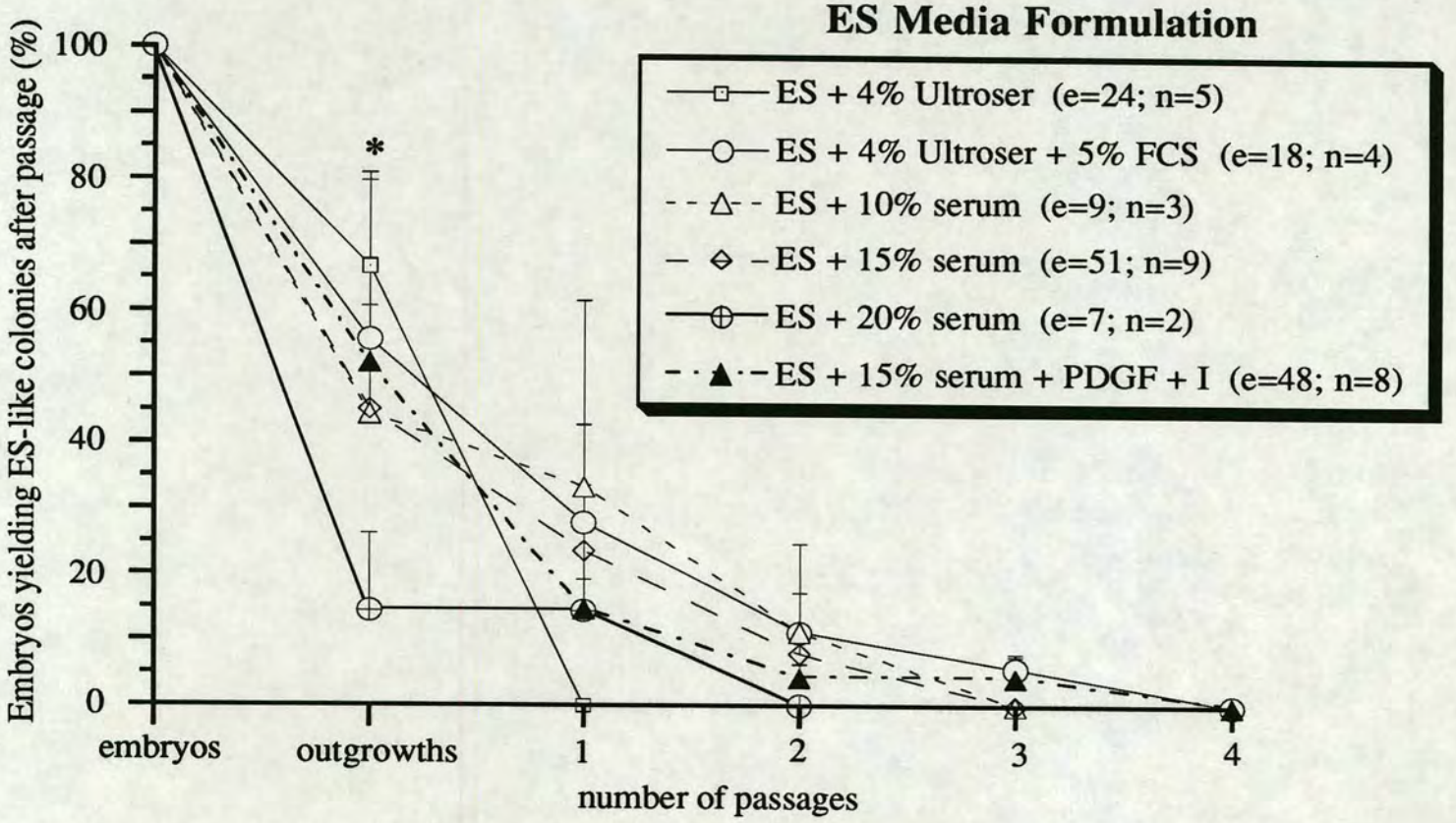

Figure 7.4: The survival profiles showing the effect of media formulation on the percentage of ovine embryos giving rise to ES-like outgrowths and colonies after repeated passage in culture.
(mean ± s.e.m.; e = embryos; n = replicates) * P<0.005 ES + 4% Ultroser *cf.* ES + 20% serum

yielding ES-like colonies at each passage in different media formulations. There were no interactions between media and embryo stage of development and approximately equal proportions of embryos from each of the three blastocyst-stages were explanted into each of the media formulations.

The only significant difference between the six media formulations was in the greater proportion of embryos giving rise to ES-like outgrowths in ES_{US} medium (16/24=66.7 ± 14.2%) than in ES_{20} medium (1/7=14.3 ± 11.9%; $P<0.005$). This was due largely to the observation that embryo attachment appeared to be increased in the ES_{US} medium. Despite the high occurrence of ES-like outgrowths in ES_{US} medium, first passage colonies were morphologically unstable and all colonies rapidly differentiated (figure 7.4) Although ES_{US} medium was unable to support ES-like colonies, this medium was adequate for the establishment of epithelial-like cell lines. Apart from ES_{20} and ES_{US} media, there were no significant differences between other media formulations in capacity to (temporarily) support the growth of ovine ES-like cells. However, the only media formulations which supported ES-like colonies to the third passage were ES_{US+FCS} and $ES_{15+PDGF+I}$ media (1/18=5.6 ± 2.3% and 2/48=4.2 ± 3.2%, respectively; figure 7.4). All of these third passage colonies were derived from day nine embryonic discs (see section 7.3.1).

7.3.3 The Effect of Heat Shock and Puromycin Treatments on the Isolation of Ovine ES-like Cells

The survival profiles of ES-like colonies derived from ovine embryos which had been either heat shocked or incubated briefly in medium containing puromycin, are compared to control embryos (cultured in ES_{15}; data from section 7.3.2) in figure 7.5. The results have been collated from ES-like outgrowths and colonies derived from day seven, eight and nine blastocyst-stage embryos exposed to any one of the three heat shock temperatures (41, 42, or 43°C) or, either of the two puromycin medium concentrations (25 or 75 μg/ml). No interactions were observed between embryo stage of development and heat shock temperature or puromycin concentration. Approximately equal proportions of embryos from each of the three blastocyst-stages were explanted into each of the treatment groups.

Although there appeared to be a trend for a slight increase in the proportion of embryos giving rise to ES-like outgrowths with both heat shock (45/69= 65.2 ± 6.9%) and puromycin (12/16=75 ± 0%) compared to control (day seven to day nine) embryos (23/51=45.1 ± 5.8%; $P>0.05$) there were no differences in the proportion of embryos yielding ES-like colonies at the first passage (figure 7.5). ES-like colonies derived from either heat shocked or puromycin-treated embryos and

cultured in ES_{15} media, like colonies derived from control embryos, did not maintain a stable ES-like cell morphology for more than two passages *in vitro*, regardless of the stage of embryo development (figure 7.5). However, cultures derived from heat shocked embryos comprising epithelial-like cells have been maintained for at least 10 passages.

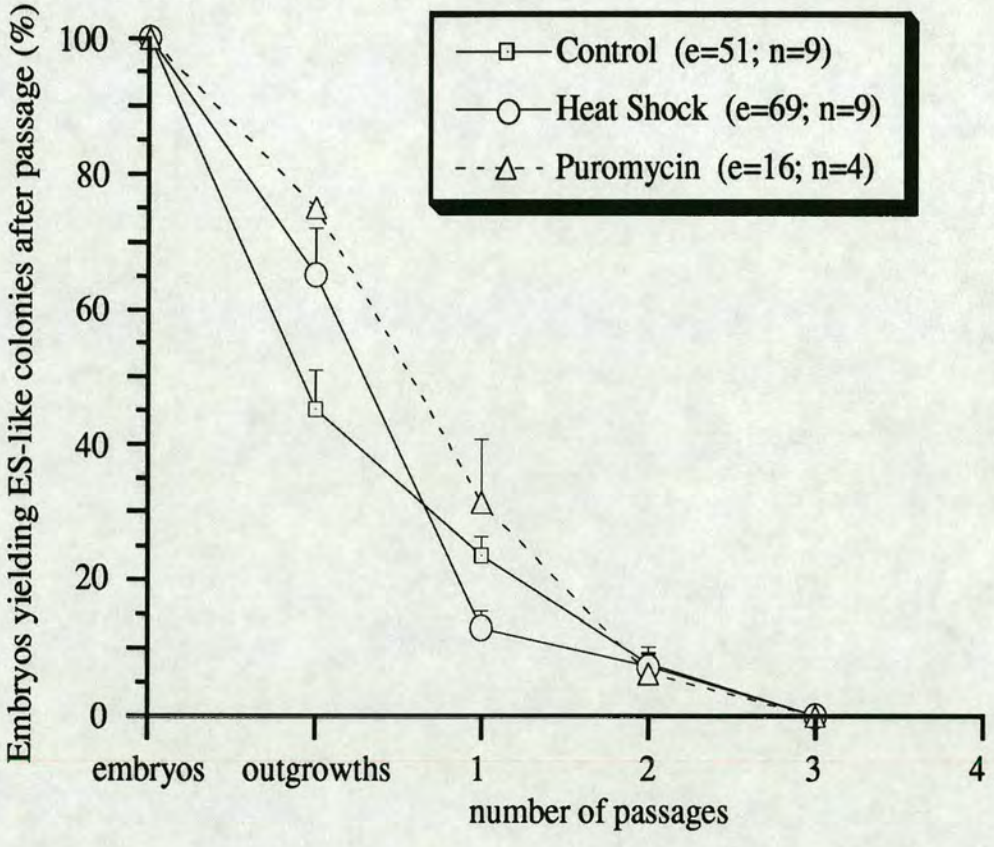

Figure 7.5: The survival profiles showing the effect of heat shock and puromycin treatments on the percentage of ovine embryos giving rise to ES-like outgrowths and colonies after repeated passage in culture.
(mean ± s.e.m.; e = embryos; n = replicates; P>0.05)

7.4 DISCUSSION

Cells possessing an ES-like morphology, similar to that described by Notarianni and colleagues (1990a), were observed here in cultures derived from day seven to nine

sheep embryos, however, they could not be maintained for more than a maximum of three passages (approximately four weeks) *in vitro*. These ovine ES-like cells also showed characteristic slow cellular growth and the cells either eventually senesced or were morphologically unstable and progressively differentiated, typically into an enlarged cell phenotype. These findings are similar to those observed elsewhere for the sheep (McWhir *et al.*, 1991; Piedrahita *et al.*, 1990b).

Comparing the culture techniques utilised in this study to those that have been employed successfully to isolate and stably maintain an ovine ES-like cell line (Notarianni *et al.*, 1990a; 1991) it is not obvious which factors may be critical in the establishment of permanent cultures of ovine stem cells. This discussion compares the culture system used here to those that have been described elsewhere. Five particular factors are known to be different and will be discussed in turn: namely, the effects of incubator temperature, media, feeder cell layers, stage of embryo development and method of culture.

Porcine and ovine ES-like cells have successfully been isolated and maintained *in vitro* at 37°C (Dr E. Notarianni, *pers. comm.*). Here, ovine embryos were cultured at 38.5°C which was closer to the normal body temperature of the ewe (39°C; Altman and Dittmer, 1968). The incubator temperature employed in these studies was a compromise, as cattle embryos were being cultured over the same period (Dr. J. McWhir and D.N. Wells, unpublished results) and the cow has a slightly lower body temperature (38.5°C; Altman and Dittmer, 1968). In our laboratory at IAPGR, there does not appear to be any difference in the survival of ES-like colonies derived from ovine embryos cultured at either 37°C (McWhir *et al.*, 1991) or 38.5°C (here).

The medium utilised by Notarianni and colleagues (1990a) was similar to the ES_{15} formulation used in these studies, however, in their medium the osmolarity was not reduced and would have been approximately 335mOs (see chapter 2.1.2). Although preliminary findings suggest no difference in murine ES cell isolation with varying media osmolarity (Wells, results not presented) the use of 290mOs medium resulted in superior plating efficiency with an established murine ES cell line (McWhir, *pers. comm.*). It is appreciated that substantial differences exist between different batches of sera in their capacity to support the proliferation of stem cells (Robertson, 1987). While the sera used in this study were selected for the growth of murine ES cells, the same sera may not necessarily be suitable for the isolation of stem cells from other species. However, the batches of NCS and FCS used in these studies have maintained the ovine ES-like line isolated by Elena Notarianni (Dr. J. McWhir, *pers. comm.*) while the serum used by Notarianni has not been successful in extending the culture of ovine ES-like cells beyond the third passage in our laboratory at IAPGR

(McWhir *et al.*, 1991). This suggests that other factors in the culture system are important.

In the studies here, only ES_{US+FCS} and $ES_{15+PDGF+I}$ were capable of maintaining ES-like colonies to the third passage. Embryo attachment was poor in ES_{20} medium, possibly due to the high serum content (Betteridge and Flechon, 1988). Interestingly, attachment was promoted in medium containing 4% Ultroser (ES_{US}) which at this concentration is equivalent to 20% FCS (Product Information; Life Technologies). However, Ultroser may not have many large molecular weight proteins; thus, making the embryos more adhesive in ES_{US} than in high serum-containing medium. Despite the high rates of attachment and embryo outgrowth, ES_{US} medium was not able to maintain colonies with an ES-like cell morphology without differentiation. It may be appropriate to promote embryo attachment in ES_{US} medium before transfer to other medium more suited to the growth of ES-like cells. ES_{US} medium was, however, satisfactory for the growth of epithelial-like colonies. The survival profiles of ES-like colonies derived from embryos grown in serum-containing media were very similar, except for medium containing 20% serum where embryo attachment was poor and differentiation was promoted. The significant effects of varying media composition upon embryo attachment and outgrowth confirm that this is an important area for further investigation.

While the addition of exogenous growth factors has not been necessary for the establishment of ovine and porcine ES-like cell lines (Notarianni *et al.*, 1990a), such factors may still have some beneficial effects. ES_{15} medium containing PDGF and insulin supported the growth of ES-like colonies for longer, than just ES_{15} medium. The addition of murine recombinant DIA/LIF to medium has been observed to increase the frequency of ovine ES-like colonies at the first passage, however, DIA/LIF was not capable of preventing the subsequent differentiation of these colonies (McWhir *et al.*, 1991). There may be a species specific requirement for this anti-differentiation factor. The ovine DIA/LIF gene has apparently been cloned and sequenced (Gough *et al.*, 1989), however, it has not been reported whether the recombinant DIA/LIF factor has been produced *in vitro*. It may be useful to include ovine DIA/LIF in the medium. In order to be able to isolate stem cells from the sheep, it may be necessary to consider experiments in which embryos (or ICMs) are cultured in medium with different concentrations of serum, supplemented with a variety of growth factors each at several different concentrations.

The choice of feeder cells in the co-culture system may have an important influence on the capacity to derive stem cells. The roles of the feeder layer may be in providing a suitable substrate for cell attachment and in producing diffusible and/or extra-cellular matrix-associated growth and/or anti-differentiation factors (Martin and

Evans, 1975; Rathjen *et al.*, 1990b). In a comprehensive study on the effect of several feeder cell types, porcine ES-like cells could only be established on feeder cell layers prepared from the STO mouse fibroblast cell line (Piedrahita *et al.*, 1990a). In the sheep, however, it was reported by the same group that colonies with an ES-like morphology were transiently observed only with ovine embryonic fibroblast cell feeders and not with STO cells (Piedrahita *et al.*, 1990b). However, STO feeder cell layers (which were employed in these studies) have been utilised successfully in the isolation of an ovine ES-like cell line (Notarianni *et al.*, 1990a).

In the studies reported here, the zona pellucida was removed from early blastocyst-stage embryos because it had been observed initially that many embryos failed to hatch from the zona or, at best, embryo hatching was a prolonged process, in the STO co-culture system. This prolonged hatching phase, combined with the prolonged period before attachment to the feeder cells with intact blastocysts, often appeared to result in the degeneration of the ICM. Compared to STOs, porcine uterine epithelial cell feeder layers have been shown to promote embryo development and hatching in the pig, however, unlike the STO cells, the uterine epithelial cells were not satisfactory for embryo attachment (Piedrahita *et al.*, 1990a).

It seems unlikely that any of these three factors (incubator temperature, media, or feeder cell layers) accounts for the failure to isolate ES-like cells from the sheep in the studies described here, compared to other workers (Notarianni *et al.*, 1990a; 1991). Other factors which may be important are embryo stage of development and interactions between embryos in culture.

The differences between the four embryo stages examined here, in the proportions of embryos giving rise to ES-like outgrowths and then to first passage colonies, were possibly due more to differences in the way the embryos were treated before culture, rather than actual embryo stage differences. In day nine embryos, the embryonic discs were dissected away from the trophectoderm before being explanted *in vitro*. In day seven and eight blastocysts, the ICM was too small to be manually dissected and immunosurgery was generally unsuccessful with the J104 and anti-*I* anti-sera. So, these blastocyst-stage embryos were normally explanted intact. Nearly all embryonic discs (and ICMs) attached within a short time to the STOs, whereas relatively fewer intact blastocysts attached and they were also slower to do so. Similar observations have been made by Notarianni and colleagues (1991). These differences in attachment, combined with the observation that fewer intact blastocysts yielded outgrowths from the ICM (possibly because of senescence during the prolonged attachment phase) lead to the significantly lower proportion of day seven and day eight embryos yielding ES-like outgrowths, compared to day nine embryonic

discs. However, the outgrowth of an eight day old ovine intact blastocyst has given rise to an ES-like cell line (Notarianni *et al.*, 1990a; 1991).

Very few of the blastomeres from disaggregated compacting sheep morulae attached to the STOs in these studies; a problem also experienced with disaggregated mouse morulae (results not presented). It is possible that the EDTA/PBS solution used to dissociate the blastomeres may have damaged the cell membranes. While it may be easier to physically dissociate pre-compacting morulae, these blastomeres may be at a stage where their cell surfaces are not very adhesive, as indicated in the mouse at the equivalent embryo stage (Kimber, Surani and Barton, 1982).

There may have been some effects of embryo stage upon ES-like cell isolation from the sheep. Although isolated ICMs and embryonic discs attached at a similar frequency, very few ICMs gave rise to proliferating ES-like outgrowths. This may have been due simply to an influence of cell number, whereby a greater number of ICM cells may have resulted in a greater chance of propagating those cells of a stem cell lineage. If this was the case, then several early cleavage-stage sheep embryos could be aggregated together and cultured to produce "giant blastocysts", from which a larger ICM component could be isolated for stem cell culture.

The isolation of ES cells may depend upon appropriate interactions between the embryos in culture. While ES cells condition medium and promote their own growth (Martin, 1981), the presence of differentiated cells may stimulate ES cells to differentiate (Robertson, 1987). The culture conditions, such as density of embryos and frequency of cell passage, may have significant effects upon these inter-relationships.

In these studies, intact embryos and embryonic discs were explanted initially in groups, onto STO feeder layers prepared in 1ml tissue-culture wells, to allow any factors produced by the embryos or outgrowths, and in particular the ICM/primitive ectodermal cells, to have co-operative effects and stimulate the overall growth of undifferentiated cells in the culture. It may be useful to vary the number of embryos in an individual culture well in order to define the optimum. By culturing murine pre-implantation embryos in groups in microdrops, both development and cell division were increased (Paria and Dey, 1990). This co-operative effect of growth factors may be dependent upon the number of embryos cultured and in what volume of medium. Any embryonic growth factors would be synthesised in very small amounts and by culturing embryos here in 1ml wells, any effect may have been diluted out. To avoid this effect of dilution of growth factors, embryos could be group-cultured initially in 50µl microdrops in future experiments; this would represent a 20-fold reduction in the volume of medium compared to what was used in these studies. The decision not to use microdrops in the present experiments was to avoid any possible toxic effects of

paraffin oil on sheep embryos and cell cultures. Although there may be species differences in sensitivity, this fear of toxicity may have been unjustified as paraffin oil appears to have no detrimental effects on murine ES cell isolation (this thesis).

Similar growth factor interactions may be important in later phases of ES cell isolation. It may have been beneficial to have pooled several disaggregated ICM outgrowths together, into the same feeder well, rather than culturing all the cellular pieces from one embryo outgrowth each in a separate well. This may have helped to stimulate the growth of any ES-like colonies present in a paracrine manner, as murine ES cells do appear to condition their own medium (Martin, 1981). If this approach had been adopted, then it would have not been possible to follow the fate of individual embryos. However, with the objective of isolating permanent cultures of ES-like morphology, such a strategy may be useful as shown elsewhere (Dr E. Notarianni, *pers. comm.*). If successful, the stem cells would have to be single-cell cloned, as it could not be guaranteed that the cells were derived originally from one embryo.

There may be important genetic effects upon the isolation of ES cells (see chapter three), with differences between species, strains and individual embryos. Although comparative data on the efficiency of isolation of ES-like cells between the pig and the sheep in laboratories successful in the establishment of such cells is limited, there does appear to be a difference between the two species, with porcine embryos yielding stem cell-like colonies at a higher frequency than in the ewe (Notarianni *et al.*, 1990a; 1990b; Piedrahita *et al.*, 1990b). During pre-implantation embryology, the embryos of both pigs and sheep form the characteristic quiescent embryonic disc of the ungulate species. The only obvious difference between the two species, over this period of embryo development, is in the timing of primitive endoderm differentiation; occurring relatively earlier in the sheep (on day seven) than in the pig (on day 10-12) (Handyside *et al.*, 1987; Notarianni *et al.*, 1991). If this is an important difference between the two species, accounting for the subsequent effects on stem cell isolation, then it may be necessary to culture embryos at earlier stages than were used here. If problems with embryo and cellular attachment can be overcome, with the use of more suitable feeder cells or factors which promote the adhesive properties of cells, it might then become productive to culture individual blastomeres from morula-stage sheep embryos. Such cells would not have been influenced by endoderm and may even be more mitotically active than the cells of the quiescent ICM or embryonic disc.

In the mouse, the culture of implantationally delayed embryos significantly increases the yield of ES cell lines (see chapter three). The only ungulate species to have an (obligative) form of implantational delay is the roe deer (*Capreolus capreolus*; Rowlands and Weir, 1984). The culture of a small number of delayed embryos recovered from roe deer in the wild, during the period of seasonal embryonic

diapause, has not given rise to any progressively growing ES-like outgrowths or cell colonies (Dr. J. McWhir, unpublished work). This might suggest deficiencies in the culture system and the need for species specific requirements.

As an alternative approach to minimise the effects of differentiated cells, an effective anti-serum specifically against trophectodermal and endodermal cells may be useful in both immunosurgically isolating pure populations of ICM cells from sheep blastocysts and for the specific cell lysis of differentiated cell types in early stem cell cultures. The presence of differentiated cell types appears to promote further differentiation (Robertson, 1987; and author's observations). Often ES-like colonies are too small to be picked out of cultures without extensive damage and "cell-peeling" techniques are not always effective in removing all differentiated cells. It has been observed here that once an ES-like colony commences to differentiate, firstly around the periphery of the colony, it is difficult to prevent the other cells within the colony from also being induced to differentiate. One way to prevent this is to disaggregate the colony, preferably into single-cells. However, early ovine ES-like colonies generally do not survive trypsinisation into single-cells, especially in the case of ES-like colonies with very low cell numbers. An alternative strategy might be to use an anti-serum specifically against differentiated cells, which could lyse differentiating cells and so prevent the "factors" that may induce the remaining cells of the colony to change their morphology. The use of an ovine anti-trophectoderm anti-serum produced by Notarianni and colleagues (1990a) (which apparently binds to primitive endoderm as well) may be of some use in this approach; however, it has not been reported that this anti-serum does not bind to epitopes on the ICM. The B006 anti-carbohydrate antibody specifically recognises determinants present on the cell surface of the trophectoderm and (to a more limited extent) the primitive endoderm (see chapter six) and may be of potential use for immuno-cell lysis. A difficulty would arise in this system if the antibodies also bound to determinants present on the feeder cells.

The inability of heat shock and puromycin treatments to result in the maintenance of ovine ES-like cells for more than two passages, may reflect a fundamental species difference compared to the mouse and/or, unfavourable culture conditions. Heat shocked and puromycin-treated embryos were cultured in ES_{15} medium, which was found in contemporary experiments not capable of supporting ES-like colonies derived from control embryos for more than two passages either. ES_{15} medium supplemented with PDGF and insulin or, medium containing Ultroser and FCS, may have been (slightly) more suitable.

With sheep embryos, it is not known what temperature increase is required to result in the induction of the HSPs . In species such as the mammals, the maximum heat shock response is typically achieved when cells are exposed to a temperature

approximately 5°C above the physiological norm (Lindquist, 1986). However, the length of time the temperature remains elevated dictates the degree of stress the cells experience and in turn, the level and duration of production of the HSPs (DiDomenico *et al.*, 1982). As the body temperature of the sheep is 39°C (Altman and Dittmer, 1968) it is possible that the 10 minute exposure of ovine embryos to the maximum temperature used in these studies of 43°C was neither sufficiently high enough nor long enough to stress the cells into releasing the HSPs, or at least, not for any extended period of time. In future experiments, the brief exposure of sheep embryos to temperatures in the range of 44-46°C may be more appropriate in inducing greater production of the HSPs and perhaps also, in producing a stimulatory effect on the isolation of stem cells as observed in the mouse (chapter five). When reliable methods for the isolation of ungulate ES cells are available, it remains to be determined whether or not heat shock or puromycin treatments are effective in these species.

The heat shock effect on stem cell isolation may be related to the stage of embryo development. In particular, the timing of heat shock in relation to endoderm differentiation may be very important. Although not significant, there appeared to be a slight increase in the proportion of mouse embryos yielding ES cell lines following heat shock of expanded blastocysts compared to hatched blastocysts (see chapter five), If the heat shock effect operates by interrupting the expression of genes responsible for differentiation of the ICM (see chapter five), one might want to impose the heat shock before any primitive endodermal cells have begun the process of delaminating from the ICM. Of the sheep blastocysts treated with heat shock or puromycin in these experiments, only some of the day seven embryos would have been expected to have had no endodermal cells present (Handyside *et al.*, 1987; McWhir *et al.*, 1991). Embryo recovery on day six would yield some early blastocysts without endoderm, but given that attachment of intact embryos tends to be poor, with the ICM degenerating within the embryo over time and that isolated ICMs appear to be too small to proliferate actively in culture, such an approach may be not be very fruitful. Perhaps a more useful stem cell isolation strategy would involve the aggregation of several eight-cell embryos together, to produce giant morulae, which were then heat shocked just after the embryos had begun to cavitate (so as to ensure that no endoderm was present) followed by immunosurgery, to isolate the enlarged ICMs for further culture.

In summary, with the treatments studied here, the micro-dissection of day nine embryonic discs gave rise to higher proportions of ES-like outgrowths and first passage colonies which tended to survive and maintain an ES-like morphology slightly longer in culture, than intact blastocysts or ICMs from earlier embryo stages. This increase with embryonic discs may have been due to the higher rates of attachment,

higher numbers of ICM/primitive ectodermal cells and to the removal of at least the differentiated trophectodermal tissue from the cultures. If these points are important, then the immunosurgical isolation of ICMs derived from "giant blastocysts" at an early stage, when the ICM may be less influenced by primitive endoderm, may be productive. Although medium similar to one formulation tested here, has been successful in the stable maintenance of one ovine ES-like cell line (Notarianni *et al.*, 1990a), culture conditions need to be improved for ovine embryos and for the ungulate species in general. Medium supplemented with PDGF and insulin appeared to maintain ES-like colonies for slightly longer. Factors preventing differentiation such as ovine LIF may also be beneficial in maintaining the ES-like phenotype. Mass explant cultures of pure ICM tissue, in a small volume of medium, may have auto-stimulatory effects by concentrating any growth factors and/or anti-differentiation factors produced by the ICMs themselves. Such culture conditions may be important in stimulating the growth of ES-like cells derived from tissues which are mitotically inactive *in vivo* in the ovine embryo. Despite species differences in the factors which may be necessary to derive pluripotent cell lines from early mammalian embryos, the mouse still provides a model animal in which to study the mechanisms of stem cell isolation, which might aid their establishment in other species.

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